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Research on the Genetic Mechanism of Leukemogenesis (Chromosome in the Human-and Mouse-leukemia and Virus-like Particles in the Leukemic Tissue)

By

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(Received for publication, October 20, 1960)

INTRODUCTION

Accurate knowledge concerning the chromosome in leukemia is still meager, when it seems to be of real importance to make clear the genetic mechanism of leukemogenesis. Recently we have studied the chromosomes in human- and mouse-leukemia and made a further investigation into the normal somatic chromosomes in order to compare the normal complex with the chromosome condition in leukemic cells.

Moreover, we have performed a search of virus within the leukemic tissue in the human- and mouse-leukemia to throw light on one of the causes of the change in karyotype of leukemia.

EXPERIMENTAL

The Material, and the Method of Examination

The human leukemic- and somatic-cells used for observation were obtained by puncturing the bone-marrow of the patients of myelogenous leukemia and that of the normal human adults. The mouse leukemic- and somatic-cells suspended in the spleen-or lymphgland-immersion and in the peripheral blood were used for microscopic observation.

To the small amount of this material used for observation a hypotonic solution (a mixture of equal volume of N/1000 NaCl and N/100 $CaCl_2$ solution) was added on slide, and having been left alone about 5 minutes, these materials were squashed with an acetic orcein solution (1% orcein dissolved in 40% acetic acid) and the chromosomes were investigated in reliable metaphase plate, and chromosome counting and the karyotype analysis were made from this metaphase plate. Doubtful metaphase plates which might induce erroneous counting were not used for investigation. The mouse leukemia of our subjects of investigation,

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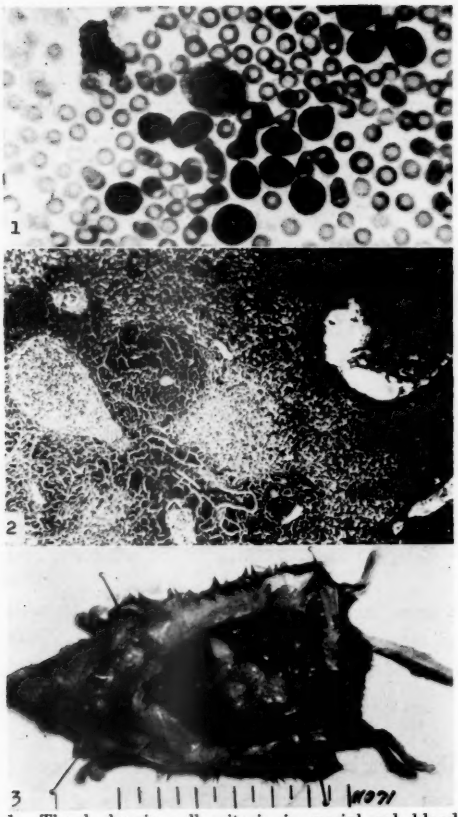


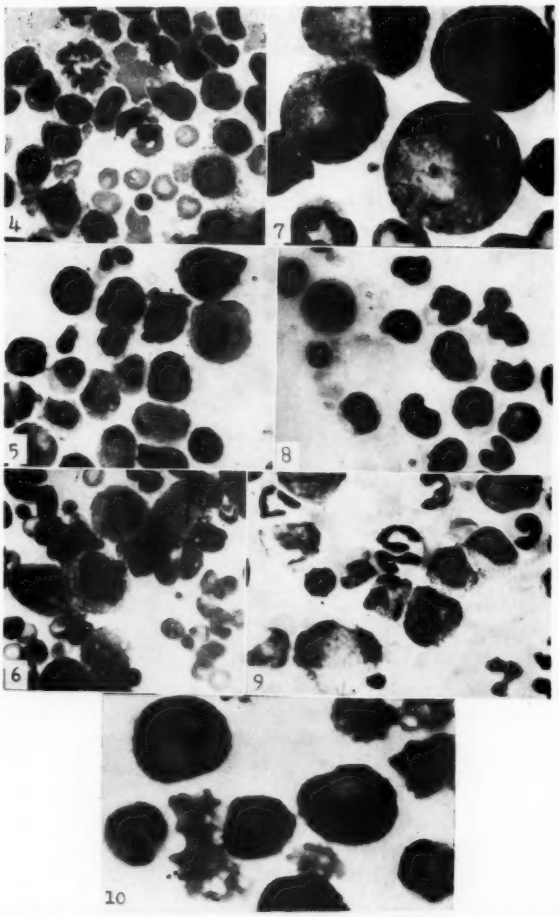
Fig. 1. The leukemic cell mitosis in peripheral blood of our lymphoid mouse leukemia.

Fig. 2. Section of liver in lymphoid mause leukemia. Leukemic infiltration of the Glisson's capsule to be seen.

Fig. 3. Observation of abdominal section of our mouse leukemia. Enlarged liver, spleen, and lymphnodes are observed.

was spontaneous leukemia which occurred in a mouse of C58 substrain. The microscopial observation of peripheral blood smear dyeing, the macroscopical observation of abdominal section and the histological structure of liver tissue of this mouse leukemia are shown in Figs. 1–3.

The cases of human leukemia, subject of investigation, were five cases of acute and subacute myelogenous leukemia and four cases of chronic myelogenous leukemia. The microscopical observation of bone-marrow smear dyeing of these



Figs. 4–10. Bone marrow smears with Giemsa-dyeing in the case A. B, C, E, G, H, and I. Myeloid cells are recognized in these photomicrographs.

cases of leukemia are shown in Figs. 4–10.

The virus is searched in the lymphgland tissue of mouse leukemia and in the liver tissue of a patient of chronic myelogenous leukemia by means of electronmicroscope observation.

THE RESULTS AND REMARKS

1) The chromosome of mouse leukemia in comparison with normal mouse complex.

The chromosome number in every somatic cell of C58 mice is determined as 40 in both sexes though there are found a few cells with polyploid number of chromosomes.

The chromosome pattern of somatic cells of normal mice is found to be entirely composed of rod-shape chromosomes and the Y-element is the smallest rod-shape one, but the X-element remains unexplained at present (Figs. 11–12). But in one of polyploid cells we have found two J-shaped chromosomes as shown

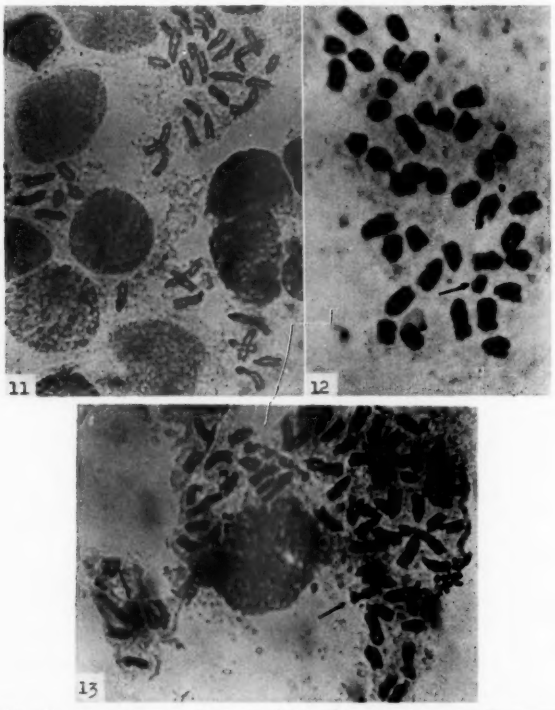


Fig. 11. Somatic complex in spleen of a normal mouse is composed of rod-shaped chromosomes.

Fig. 12. Showing metaphasic gonad cell mitosis of a normal male mouse. Heteropyknotic Y-chromosome is indicated by an arrow.

Fig. 13. Near tetraploid cell of a normal mouse contains two J-shaped chromosomes indicated by arrows.

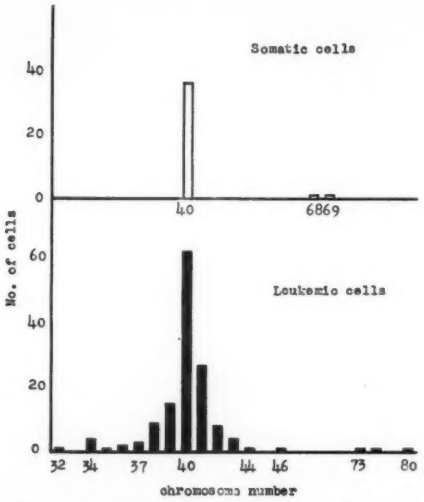


Fig. 14. Chromosome number distributions of somatic cells, and leukemic cells in C58 substrain mice based on Table I.

TABLE I. Variation of Chromosome Numbers in Somatic Cells and Leukemic Cells of C58 Mice.

No. of chromosomes	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47..68	69	73	75	80	No. of cells observed
Somatic cells									36								1	1			38
Leukemic cells	1		4	1	2	3	9	15	63	27	8	4	1		1				1	1	142

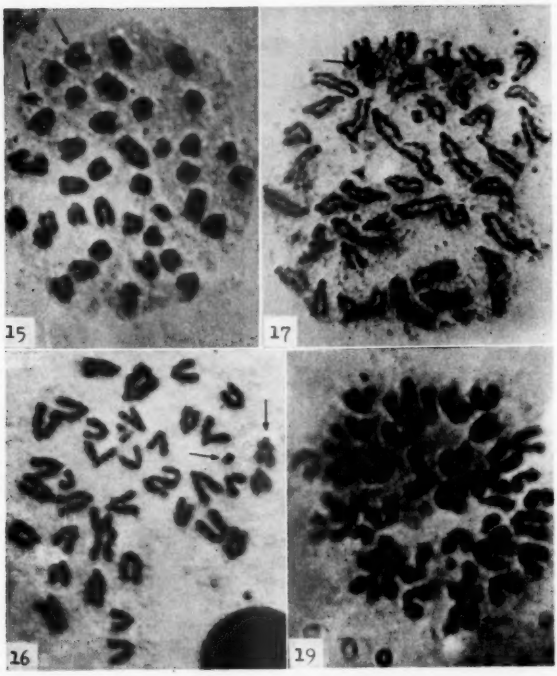
in Fig. 13. In our mouse leukemia the basic chromosome number was also 40, but it showed a variation ranging from 32 to 46, and besides, there were a few polyploid cells. (Fig. 14, Table I)

The chromosome pattern in our mouse leukemia is also found to be entirely composed of rod-shaped chromosomes, and chromosome of the stemline cells in our mouse leukemia is nearly identical both in number and in pattern with the somatic chromosome in normal mice.

However, the detailed comparison of the chromosome in the leukemic cells with the somatic chromosome has revealed the following remarkable facts.

First, in the leukemic cells a dotlike chromosome (very short rod-shaped one) which is never found in somatic cells of normal mice is to be seen as shown in Figs. 15–16.

Second, in most cases one of the medium-sized rod-shaped chromosomes undergoes a changes, that is to say, it does not get stained with orcein at the



Figs. 15–16. Leukemic peripheral blood cells in metaphase contain two marker chromosomes indicated by arrows. Fig. 17. The medium-sized marker chromosome is heteropyknotic in late prophasic cell. An arrow indicates the marker. Fig. 19. A cell in mouse leukemia having a J-shaped chromosome.

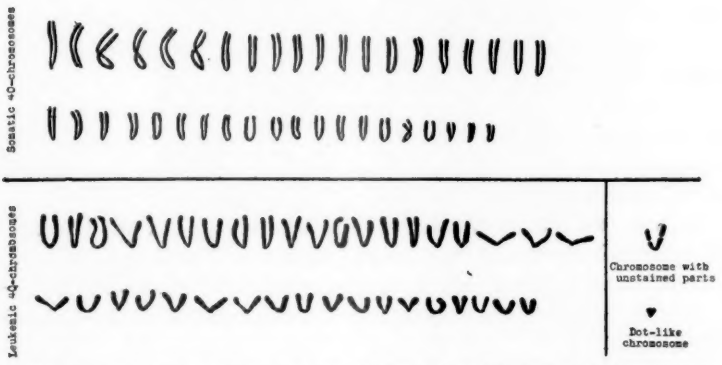


Fig. 18. Showing idiograms analysis of somatic, and leukemic 40-chromosomes. Two particular chromosomes of leukemic cell are distinct.

intermediate part of the arm of this chromosome and so the arm of this chromosome looks as if it were cut in two.

However it is not get made clear whether this portion of chromosome has undergone a change in chemical structure or whether the fragmentation has really occurred here (Figs. 15–16). Nor are we clear whether this chromosome is the X-element or not, but this chromosome is especially more thickly dyed by acetic orcein in late prophase plate than any other chromosomes as is seen in Fig. 17. And so this may be the X-element but this is still undetermined.

Fig. 18 shows the idiograms of our mouse leukemia in comparison with those of normal mice.

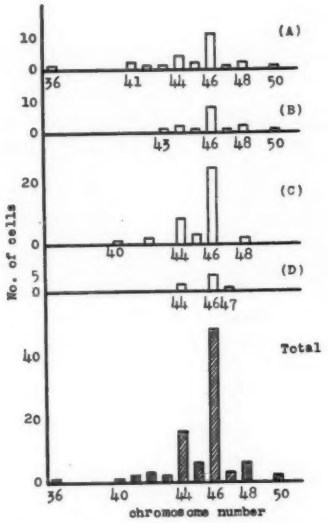
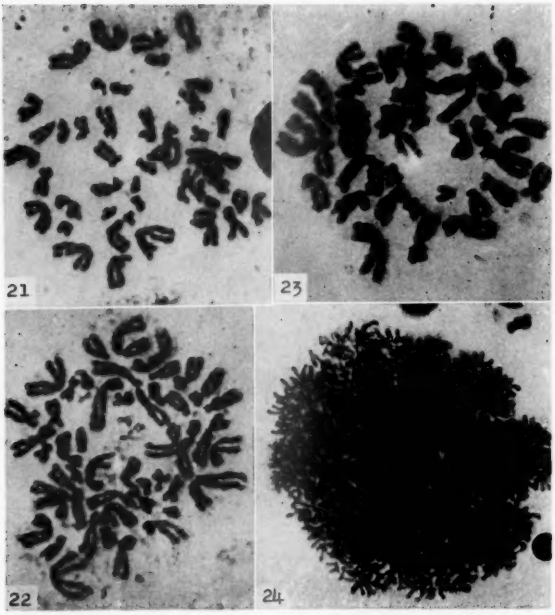


Fig. 20. Chromosome number distributions of four normal individuals based on Table II.

TABLE II. Variation of Chromosome Numbers in Somatic Cells of Four Normal Human Adults.

Sex	Individuals	No. of chrom.															No. of cells observed
		36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	
Males	(A)	1					2	1	1	4	2	11	1	2		1	26
	(B)								1	2	1	8	1	2		1	16
	(C)					1		2		8	3	24		2			40
Female	(D)									2		5	1				8
Total		1				1	2	3	2	16	6	48	3	6		2	90



Figs. 21–23. A human male somatic cells.
21. Diploid cell having 46–chromosomes.
22. Heteroploid cell having 47–chromosomes.
23. Heteroploid cell having 44–chromosomes.

Fig. 24. Polypolar division in normal human bone marrow.

Third, we found that there are a few cells in mouse leukemia which comprise a peculiar large J-shaped chromosome (Fig. 19), but they are very few in number and recently we have found this chromosome in a polyploid cell from embryonic tissue of a normal mouse (Fig. 13).

Therefore, the significance of this chromosome is not still understandable and this may not be characteristic of malignant growth.

2) The chromosome of human myelogenous leukemia in comparison with the normal human complex.

a) Chromosome of normal somatic human complex

Recently we have made a report of the chromosome of somatic human bone marrow cells in 1958 and disclosed that the chromosome number of human somatic cells is determined as 46, though there is a variation ranging from 40 to 50 and this result is in agreement with the findings of Tjio and Levan (1956), Ford and Hamerton (1956), Makino and Sasaki (1959). (Fig. 21)

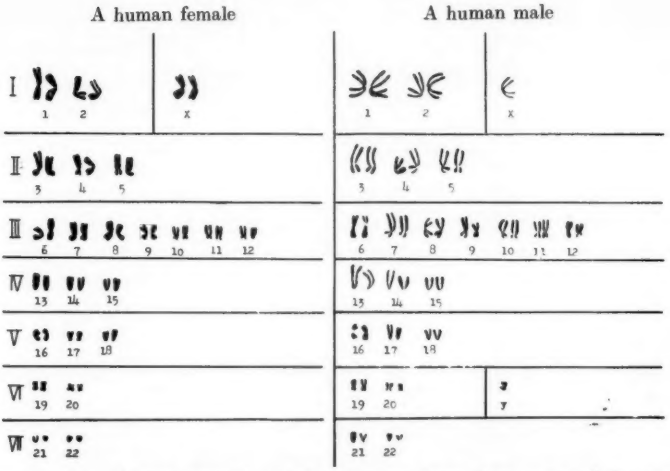


Fig. 25. Human somatic 46-chromosomes have been grouped in seven classes on the basis of their sizes and the positions of their centromeres.

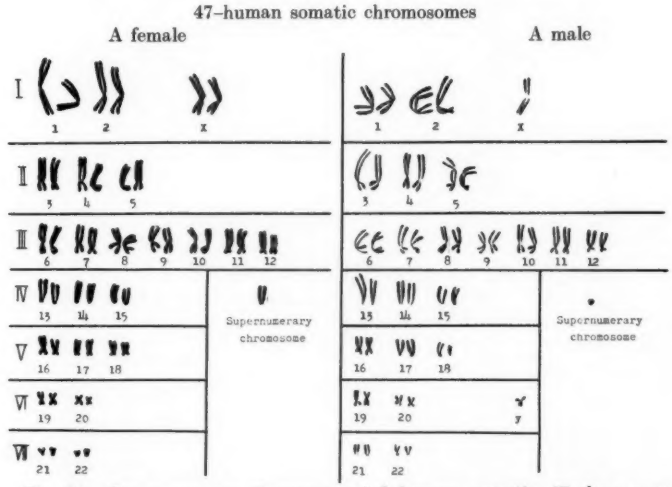


Fig. 26. Supernumerary chromosome of human somatic 47-chromosomes is rod-shaped one.

Fig. 20. and Table II show the frequency distribution of the chromosome number of somatic complex with a diploid number (46) of chromosome.

Fig. 22 shows the hyperdiploid cell having 47 chromosomes, and Fig. 23 shows the hypodiploid cell having 44 chromosomes. Besides, there are found a

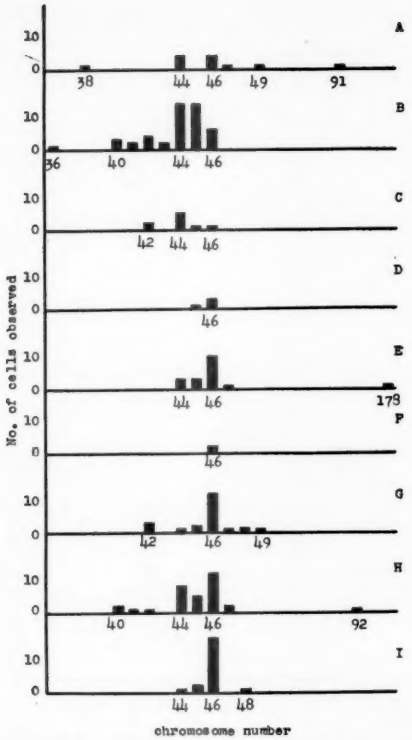


Fig. 27. Chromosome number distributions of bone marrow cells in myeloid leukemic nine patients set up with the data from Table III.

TABLE III. Variation of Chromosome Numbers in Bone Marrow Cells of the Nine Cases of Myeloidleukemia.

Sex	Patients	No. of chrom. 36	37	38	39	40	41	42	43	44	45	46	47	48	49	..91	92	..178	No. of cells observed
Males	A : acute			1						4		4	1		1	1			12
	B : subacute	1			3	2	4	2	14	14		6							46
	C : subacute						2		5	1		1							9
	D : subacute									1		3							4
	E : chronic								3	3		10	1					1	18
	F : chronic											2							2
Females	G : subacute							3		1		2	12	1	1	1			21
	H : chronic				2	1	1			8		5	12	2			1		32
	I : chronic									1		2	17		1				21
	Total	1		1	5	3	10	2	36	28		67	5	2	2	1	1	1	155

few cells in the normal bone marrow which show high polyploidy and polypolar division as is shown in Fig. 24. Since the heteroploidy and polyploidy are to be observed even in the somatic cells of adult persons, such a phenomenon is not always characteristic of the malignant growth.

The idiogram analysis reveals that normal somatic human complex is aligned into 22 paris of homologous chromosomes and a pair of particular elements which are unequal in size in male cells, but equal in female cells. It is then evident that these two are the sex determining elements, and the large L-shaped chromosome having submedian centromere represents the X-element, while the smaller one does the Y-element.

The 22 autosomal pairs consists of one large pair of median V-shaped chromosomes and one large pair of submedian L-shaped ones (I group), three pairs of subterminal J-shaped one (II group), seven paris of the medium sized L- and

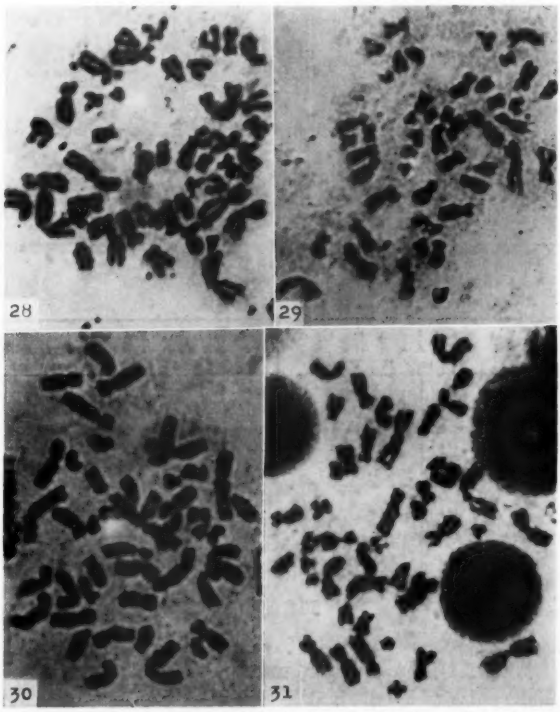


Fig. 28. 44-chromosomes of the leukemic stemline cell in the case C.

Figs. 29–31. 46-chromosomes of the leukemic stemline cells in the case B, H, and G.

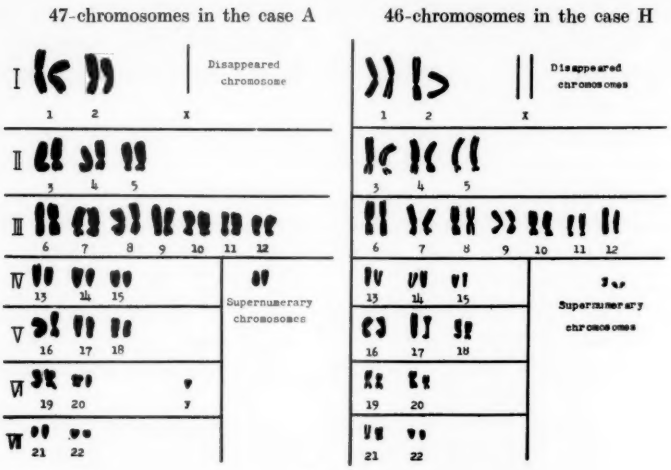


Fig. 32.

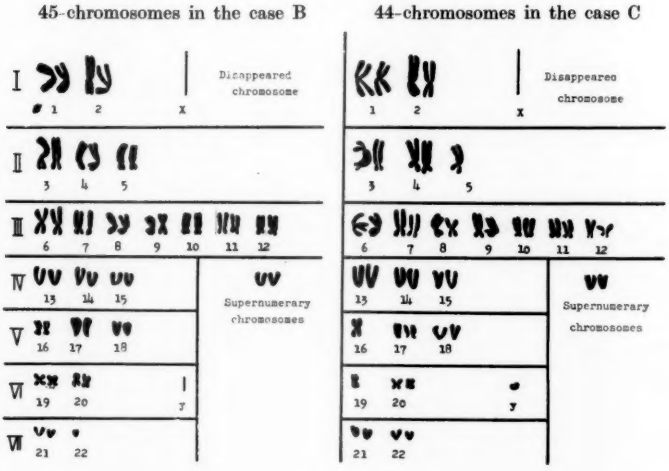


Fig. 33.

J-shaped one (group III), three pairs of medium-sized acrocentric rod-shaped one (group IV), three pairs of small-sized L- and J-shaped one (group V), two pairs of small V-shaped one (group VI) and two pairs of small-sized rod-shaped ones (group VII). (Fig. 25).

Even in the hyperdiploid cells having 47 chromosomes, the idiograms is the

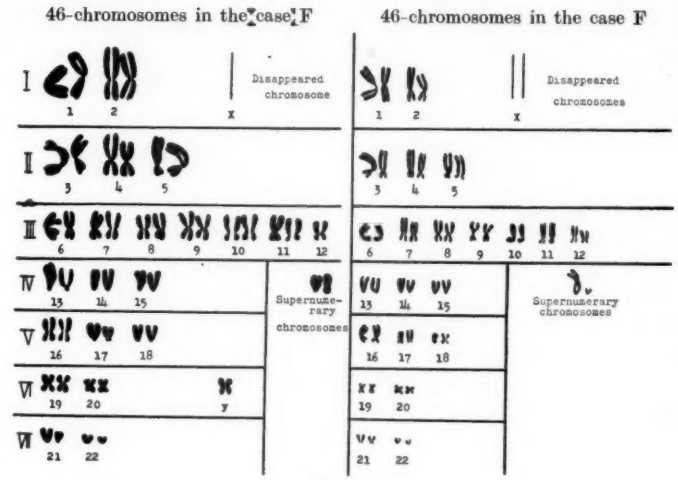


Fig. 34.

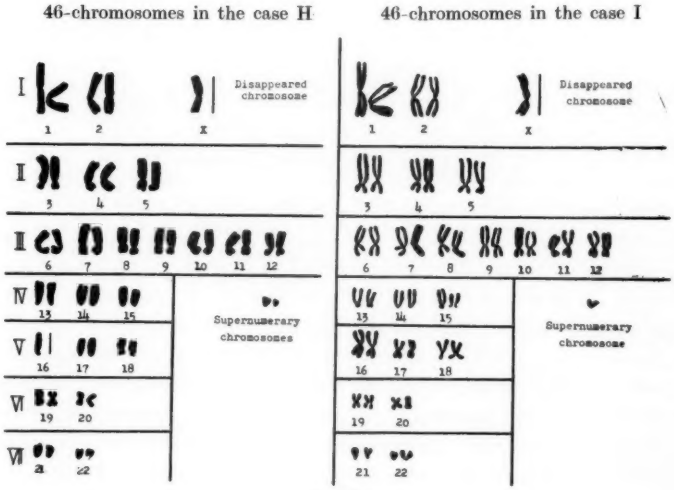


Fig. 35.

Figs. 32–35. Idiograms of the human leukemic cells. Showing disappeared chromosomes in solid lines.

same as that of diploid cells, and only one additional small- or medium-sized rod-shaped one is observed (Fig. 26).

b) The chromosome of human myelogenous leukemia.

We made a minute investigation into the chromosome of leukemic cells in five cases of acute and subacute myelogenous leukemia and four cases of chronic myelogenous leukemia, and compared these with the somatic chromosome of human adults.

The chromosome count and its frequency distribution of the leukemic cells of these nine cases are shown in Fig. 27 and Table III and the variation of chromosome count is larger in range in leukemic cells than in somatic cells, but there is always present a population of leukemic cells that are characterized by a high frequency of occurrence and by a particular chromosome number, and this stemline chromosome number varies with each of the cases of the myelogenous leukemia, and this is not only 46 but also 44 or 45. (Fig. 27).

For instance, in the case A the modes at 46 and 44 are to be seen, in the cases B the stemline number is 44 and 45, in the case C cells having 44 chromosomes are highest in frequency, in the cases D the stemline numbers are 46 and in the case H that is not only 46 but 44 and 45. The above date are collected in Table III and further illustrated in Fig. 27.

But so for as the scope of the present study is concerned, chromosome of stemline cells in human leukemia is nearly identical with that of somatic human cells both in number and in pattern.

This fact is thought to be of such importance as to suggest that these cells have derived from somatic cells. Then, we examined the detailed comparison of the chromosome pattern between the normal cells and the leukemic cells.

Figs. 28–35. show the metaphasic stemline cells and these idiograms of the six respective cases of myelogenous leukemia. In these cases, the very important findings are that the large L-shaped chromosome with submedian centromere, thought to represent the X-element, disappears in most leukemic cells and in males a large L-shaped one disappears, while in females a pair of them disappear, and then the increase of rod-shaped chromosomes is very frequently to be seen.

Judging from this the disappeared chromosome is thought to be an X-element. But it has never been made clear whether an X-element has vanished from the chromosome pattern or whether part of X-chromosome is fragmentated or transformed. As for the causes of a change in karyotype such various factors can be thought of as radiation, chemical material or virus and so on. So, we have made a virus investigation to make clear one of these causes.

3) The relation between chromosome and virus in leukemia.

It was found possible to transplant our mouse leukemia within a few days and at a high rate to the normal C58 mice even by inoculating the supernatant fluid of centrifuged organ extract of leukemic mice.

Therefore, we have made a virus investigation to make clear the causes of the change in karyotype of leukemia. Consequently, we could observed virus

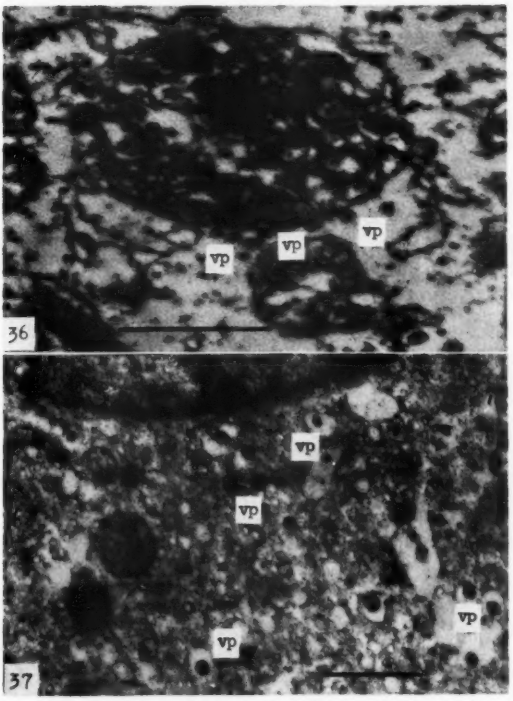


Fig. 36. Electron micrograph of virus-like particles in the lymphgland tissue of C58 mouse leukemia. VPs indicate virus-like particles.

Fig. 37. Electron micrograph of virus-like particles (VPs) in the liver tissue of the human leukemia (case H.).

like particles which are 81–118 mμ in size (average 92 mμ) in the lymphgland tissue of leukemic mice. (Fig. 36).

Then, as we thought that even in the case of human leukemia there might be some relation between such changes in chromosome and virus, we made a search for virus in the remarkably swollen tissue of liver and spleen of the patient of chronic myelogenous leukemia by means of organ biopsy and detected virus-like particles in the liver tissue of the patient.

Fig. 37 shows the electron microscopic observation of the liver tissue of this patient (case H) and we observed virus-like particles in the endoplasmic reticulum in the leukemic cells and these were 81–200 mμ (average 118 mμ) in size.

In this way we observed the virus-like particles in the leukemic tissue of the mouse leukemia and of the human leukemia, and so we can consider some

relation between the change in karyotype of leukemic cells, and the virus in leukemic tissue. Hence, there is possibility that the change perceived in the chromosome of leukemia may be due to virus.

But so far as the present study concerned these virus-like particles observed in leukemic tissue are very few in number and so this is not get determined at present.

SUMMARY

We have studied the chromosome in human- and mouse-leukemia and made a further investigation into the normal somatic chromosome in order to compare the normal complex with the chromosome condition in leukemic cells. Further, we have performed a search of virus within the leukemic tissue in the human- and mouse-leukemia to throw light on the causes of the change in karyotype of leukemia. The major conclusion obtained from the present study are summarized as follows:

1) The somatic chromosome number of normal C58 mice was 40 in every cell and the chromosome pattern was found to be entirely composed of rod-shaped chromosomes. In our mouse leukemia the basic chromosome number was also 40, but it showed a variation ranging from 32 to 43, and chromosome pattern was found to be also entirely composed of rod-shaped chromosomes.

However, in the leukemic cells the dotlike chromosome (very short rod-shaped one) which is never found in the somatic cells is to be seen, and one of the medium sized chromosome undergone a change in chemical structure, that is to say, it does not get stained with acetic orcein at the intermediate part of its arm and so the arm of this chromosome looks as if it were cut in two.

2) The normal somatic chromosome number of human adults is determined as 46 in both sexes, though there is a variation ranging from 40 to 50, and also there are found a few cells which show polyploid number and polypolar division. The normal somatic human complex consists of 22 pairs of homolognous chromosome and a pair of sex-element and the X-element represented by a large L-shaped chromosome having a submedian centromere, while the smaller one does the Y-element.

The stemline chromosome number in leukemic cells varies with each of the cases of the myelogenous leukemia, and this is not only 46 but also 44 or 45.

But the chromosome in human leukemia is nearly identical both in number and in pattern with that of the somatic human cells. However, the large L-shaped chromosome corresponding to the X-element disappears in most leukemic cells and an increase of rod-shaped chromosome is very frequently to be seen.

3) We could observe the virus-like particles in the leukemic tissue of the mouse leukemia and of a human leukemia. Hence, there is possibility that the

change in chromosome in these mouse- and human-leukemia may be due to virus, but this is not yet determined at present.

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Demonstration of Metabolic Anomaly in Congenital Nonspherocytic Hemolytic Anemia and Beneficial Effect of Vitamin B_{12} upon It

By

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Since the first description of "a new type of hereditary hemolytic jaundice without spherocytosis" by Haden in 1947[1], similar cases have been reported by several authors under various titles such as "hereditary nonspherocytic hemolytic anemia",[2][3][4] "atypical congenital hemolytic anemia",[5] "nonspherocytic chronic hemolytic anemia",[6] "familial nonspherocytic hemolytic anemia",[7][8] "chronic familial nonspherocytic anemia",[9] or "congenital nonspherocytic hemolytic anemia".[10][11]

This type of hemolytic anemia has several features in common. Jaundice or anemia usually appears soon after birth, requiring differentiation from erythroblastosis fetalis or other well-defined causes of congenital hemolytic process. Spherocytes are not found in the peripheral blood. Osmotic and mechanical resistance of the erythrocytes is normal. In most cases, no morphological anomalies of erythrocytes are observed. The anemia tends to be chronic. Hepatosplenomegaly is often present, but the course is not altered by splenectomy.[4][5]

The pattern of inheritance of the disorder is thought to be Mendelian incomplete dominance, although conclusive pattern is not yet obtained.

CASE REPORT

A 40-day-old girl was admitted to Pediatric Clinic, Tohoku University Hospital, because of severe, prolonged jaundice from 4th day of life. The family history revealed that an elder brother died of severe jaundice on 26th day after birth and that a maternal first cousin died likewise of jaundice in early infancy (Fig. 1). The parents of the cousin were consanguineous. There was no history of jaundice or anemia in the relatives on the paternal side. The parents of the patient in question were not consanguineous, and appeared to be healthy without past history of jaundice. An uneventful full-term pregnancy and spontaneous delivery were noted. No Rh incompatibility between the patient and the mother

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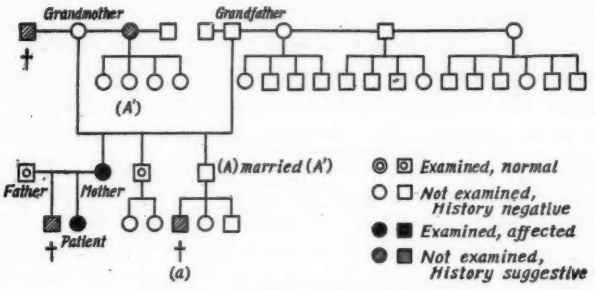

Fig. 1. Pedigree of the patient.

could be demonstrated. The patient was of type A, CDe/CDe and the mother was of type O, CDe/CDe.

Examinations showed a well developed infant who had however remarkable jaundice and hepatosplenomegaly. The liver with smooth surface was palpable 4 cm below the costal margin on the right midclavicular line, and the spleen was palpable 1.5 cm below the left costal margin. Otherwise, no abnormal findings were present.

Laboratory data: Examination of blood showed a hemoglobin of 14.6 g/100 ml, R.B.C. 3.8 M/mm^3, W.B.C. 9400/mm^3, hematocrit 36%, color index 1.25, volume index 1.03, MCV 93.0 μ^3, MCH 37.7 $\gamma\gamma$, MCHC 40.5%, MCD 7.31 μ, and MCT 2.22 μ. reticulocyte count was 42‰. No normoblasts were found in the peripheral smear. There were no spherocytes or any other morphological anomalies of erythrocytes. The differential counts of leukocytes were; lymphocytes 74%, neutrophils 20%, monocytes 3.5%, eosinophils 2.0%, and basophils 0.5%. The platelet count was 235,000. Bleeding time was 1 minute 30 seconds and coagulation time was 5 minutes. The osmotic fragility test also was within normal limits, showing initial hemolysis in 0.42% saline and complete hemolysis in 0.32% saline. Both direct and indirect Coombs' tests were negative. Wassermann's reaction of both the patient and the mother was negative. The icterus index was 94 units. The Hijmans van den Bergh reaction was negative for direct and remarkably positive for indirect. Total concentration of bilirubin in the serum was 10 mg/100 ml with the indirect fraction of 7.9 mg/100 ml. The liver function tests, including thymol turbidity test, Takata's reaction, bromsulphalein test and cobalt reaction, were all within normal limits. The urine contained no bilirubin, but increased urobilinogen. Sugar and protein were negative in urine. The feaces were yellow in color and were said to have never been acholic. Urobilinogen excretion in feaces was increased. E.K.G. and X-ray of the chest revealed no abnormalities.

Basing upon the above-mentioned results, the diagnosis of congenital non-

spherocytic hemolytic anemia was established.

Family studies: The mother showed a mild anemia, R.B.C. 3.5 M/mm³, hemoglobin 12.0 g/100 ml and hematocrit 35.5%, otherwise normal in hematological data. The father showed no hematological abnormalities.

Course of the patient: The treatment with intravenous injection of 20 ml of 20% glucose and intramuscular injection of vitamin B_{12} (50γ) were carried out daily since the establishment of diagnosis. Jaundice improved gradually and was scarecely recognized at the end of the third week of treatment. At that time, laboratory data also showed an improvement; icterus index decreased to 23 and total concentration of bilirubin diminished to 1.6 mg/100 ml with the indirect fraction of 1.0 mg/100 ml. Urobilinogen excretion in urine and feces returned to normal. These results might indicate subsidence of hemolytic process. At present, two months after the discontinuance of the treatment, the recurrence of jaundice has not been observed.

Special studies: The determination of the content of reduced glutathione (GSH) in the erythrocytes was performed by the method of Gruenert and Phillips.[12] The reduced glutathione stability of the erythrocytes was determined by incubation of whole blood with acetylphenylhydrazine (APH) as described by Beutler *et al.*[13]. The activity of glucose-6-phosphate dehydrogenase (G6PD) was assayed by the method of Zinkham *et al.*[14]. Vitamin B_{12} in blood was estimated microbiologically using Lactobacillus leichmannii.[15]

The content of GSH and the GSH stability in erythrocytes from the patient, the parents and some healthy subjects of the same age, were shown in Table I

Table I. Glutathione Studies in Erythrocytes from the Patient and the Parent.

Subjects			GSH* (mg/100ml.R.B.C.)	GSH (mg/100 ml. R.B.C.) After 2 hours of incubation with APH**
Controls	Healthy infant (1 to 2 months of age)	A	90.3	83.5
		B	89.0	87.5
		C	76.5	74.0
		D	92.5	82.5
	Healthy adult	E	67.5	62.5
		F	70.5	65.0
		G	76.2	75.0
Patient	On admission		43.6	12.8
	After VB_{12} therapy		54.0	32.5
Mother			59.5	40.8
Father			72.0	66.3

* GSH: Reduced glutathione, ** APH: Acetylphenylhydrazine.

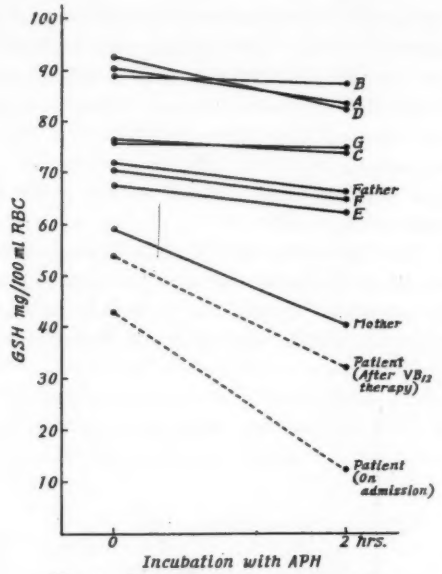

Fig. 3 The glutathione stability test.

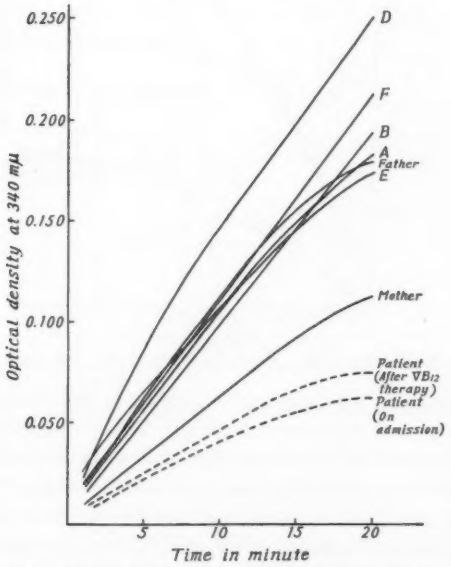

Fig. 3. Glucose-6-phosphate dehydrogenase activity.

and Fig. 2. The erythrocytes of the patient had a low level of GSH as compared with that of the controls. On the GSH stability test, GSH in erythrocytes from the patient showed a remarkable drop when incubated with APH, while GSH in erythrocytes from the controls remained almost unchanged after the incubation. The erythrocytes from the mother showed a relatively low content of GSH as well as decreased stability of GSH to a milder degree.

The erythrocytes from the father showed normal results both in GSH content and in the GSH stability test.

The results of assay for G6PD activity in erythrocytes were shown in Fig. 3. The erythrocytes from the patient showed a very low activity as compared with that from the controls. The erythrocytes from the mother showed a moderate degree of deficiency of G6PD, while those of the father were within normal limits.

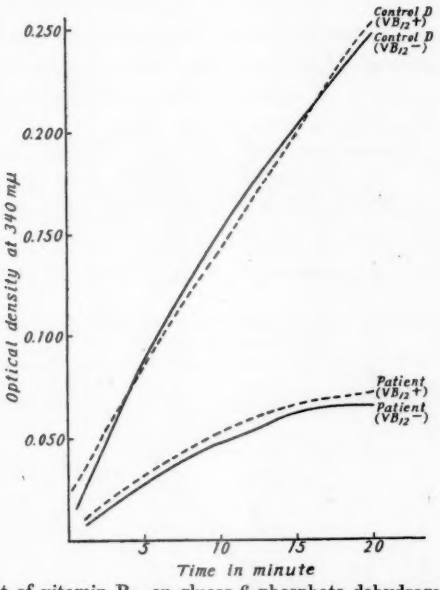

Fig. 4. Effect of vitamin B_{12} on glucos-6-phosphate dehydrogenase activity.

After the treatment with vitamin B_{12} for three weeks, GSH in erythrocytes from the patient showed an increase form 43.6 to 54.0 mg/100 ml of erythrocytes and an improvement of the GSH stability test, while the activity of G6PD remained in low level as observed in the pre-treatment stage. The concentration of vitamin B_{12} in the blood of the patient was 0.42 mγ/ml, within normal limits on admission and showed a marked rise 13.55 mγ/ml during the treatment with vitamin B_{12}.

The effect of vitamin B_{12} on the activity of G6PD in erythrocytes was investigated by adding 1γ of the vitamin to in-vitro assay system of the enzyme. The addition of vitamin B_{12} showed no significant effect on the activity of G6PD in erythrocytes from the patient as well as the control (Fig. 4).

DISCUSSION

Recently a new field of research has been developed by the studies on the metabolism and enzymatic activity of the erythrocytes in regard to the pathogenesis of hemolytic process. Beutler and other investigators[16)17)18)] elucidated that drug-induced hemolytic anemia was due to abnormality in erythrocyte metabolism. They found that the level of GSH in erythrocytes from drug-sensitive individuals was lower than that in normal and that GSH content in the erythrocytes showed a remarkable drop when these erythrocytes were incubated with APH in vitro.[19)20)] Furthermore, it was demonstrated by Carosn[21)] that these abnormalities of glutathione metabolism were related to a deficiency of G6PD in the erythrocytes of drug-sensitive individuals.

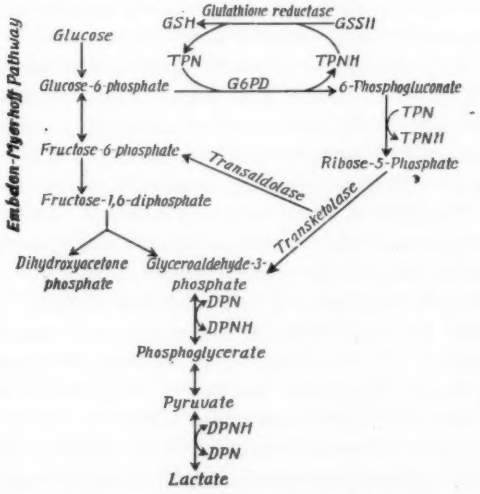

Fig. 5. Schema of glucose metabolism in erythrocytes.

The hemolytic process and its relation to these three abnormalities; low content of GSH, decreased GSH stability, and deficiency of G6PD in the erythrocytes, has been explained as follows (Fig. 5).

Although biochemical mechanism of hemolysis is not enough understood, there are several evidences showing that GSH plays an important role in the

maintenance of structural integrity of erythrocyte. Benesch[22] observed that the hemolysis induced by organic mercury compounds was protected by the previous addition of GSH in in-vitro experiments. Fegler[23] showed an inverse correlation between GSH content of horse erythrocytes and rate of spontaneous hemolysis of them. Since GSH is thought to activate a wide variety of enzyme systems, concerning carbohydrate, fat, and protein metabolism, by protecting the enzymes against their oxidation[24)25], it is reasonable to presume that GSH is essential for the viability of the cell and for the maintenance of structural integrity of cell. The fact[26)27] that glutathione exists exclusively inside the cell might support the concept mentioned above. In normal erythrocytes the maintenance of GSH is thought to be dependent on the oxidative metabolism of glucose. The conversion of oxidized glutathione (GSSG) to GSH requires the participation of reduced triphosphopyridine nucleotide (TPNH) which is generated from TPN by G6PD in the process of conversion of glucose-6-phosphate to 6-phosphogluconate. Therefore, the deficiency of G6PD results in limitation of conversion of GSSG to GSH (Fig. 5). When the individuals having a defect in G6PD in erythrocytes are exposed to a certain drug, the manifestation of hemolytic anemia is thought to take place by the marked decrease of GSH in erythrocytes as a consequence of aggravation of preexisting metabolic disturbance. The enzyme defect in drug-induced hemolytic anemia is said to be determined genetically.[28] The agents, which can induce this type of hemolytic anemia, include primaquine,[29] quinidine,[30] sulfanilamide[17], nitrofurantoin,[31] furazolidone,[32] naphthalene,[33] phenacetin.[34] and fava bean.[35] Irrespective of the kind of these drugs, the actual cause of hemolysis of sensitive individuals should be attributed to the deficit in G6PD in the erythrocytes. Larizza[36] has proposed to designate "enzyme-deficiency hemolytic anemia" for this type of the disorder induced by pharmacological or vegetable agents.

Recently, Shahidi and Diamond[11] reported that the same enzyme deficiency, as observed in the drug-induced hemolytic anemia, was found in two siblings of congenital nonspherocytic hemolytic anemia without any exposure to drugs. They described that the erythrocyte deficient in G6PD did not necessarily require the injurious effect of a drug to undergo hemolysis.

We suppose that both so-called congenital nonspherocytic hemolytic anemia and drug-induced hemolytic anemia are considered as the same entity belonging to an "inborn error of metabolism" and that the former is the case in which the manifestation of hemolysis develops soon after birth without exposure to drug and the latter is the case in which drug accentuates a preexisting metabolic disturbance to lead the manifestation of hemolysis.

We could demonstrate a lower content of GSH, its instability by incubation with APH, and a deficiency of G6PD in erythrocytes in our infant diagnosed as congenital nonspherocytic hemolytic anemia. This was the first case reported

in Japan of hemolytic anemia in which this type of enzymatic abnormality in the erythrocytes was demonstrated.

It is of interest that jaundice of our patient showed a marked improvement by the treatment with massive dose of vitamin B_{12}. Not only the improvement of clinical symptoms but a rise of GSH content and improvement of GSH instability in erythrocytes were observed in convalescence, while the activity of G6PD remained in a low level in spite of the treatment. The beneficial effect of vitamin B_{12} on hemolytic jaundice in our case might show a close relation between vitamin B_{12} and glutathione metabolism. The effect of vitamin B_{12} on the reduction of some S-S compounds to the SH-state was observed by Dubnoff[38] with in-vitro enzyme system. Ling and Chow[39] reported that the concentration of SH-compounds, chiefly GSH, in erythrocytes was increased by the administration of vitamin B_{12} in vitamin B_{12} deficient rats and in the patients with pernicious anemia. A lower content of GSH in erythrocytes was found in rats[40] or chicks[41] deficient in vitamin B_{12} and the supply of vitamin B_{12} caused an increase of GSH. Although the exact site of vitamin B_{12} on glutathione metabolism is obscure, it has been suggested that vitamin B_{12} serves to keep glutathione in the reduced form through its participation in reductive severance of –S–S– bond[38] or to incorporation of glycin into glutamylcystein in glutathione formation.[42] The deficiency of vitamin B_{12} was not recognized in our patient since the concentration in blood was within normal limits on admission. The present data, however, indicate that massive dose of vitamin B_{12} was effective for remission of hemolytic jaundice and that this effect might be attributed to an increase of GSH and an improvement of its instability in erythrocytes after vitamin B_{12} treatment. In this respect, an in-vitro experiment was made to observe whether or not vitamin B_{12} would promote G6PD activity in erythrocytes. As the result of this experiment no effect of the vitamin was recognized on G6PD activity (Fig. 4). This is in agreement with the result that the activity of G6PD remained unchanged in low level in spite of treatment with vitamin B_{12} while a marked increase of the vitamin was observed in the blood. Although the maintenance of GSH in erythrocytes would mostly depend on the activity of G6PD, it is possible that some other mechanism might take a part in the maintenance of GSH. According to Carson[42], glutathione reductase can utilize DPNH as well as TPNH as coenzyme. There is a report that glutathione reductase in primaquine-sensitive erythrocytes had an increased activity rather than normal.[43] For the explanation of the beneficial effect of vitamin B_{12} upon hemolytic jaundice observed in the present case, the following three possibilities might be supposed: 1) Vitamin B_{12} accelerates directly glutathione reductase, 2) vitamin B_{12} accelerates glutathione reductase indirectly by supplementing coenzyme through DPN dependent pathway in glycolysis, or 3) vitamin B_{12} acts on blocking the oxidation of GSH, the vitamin thus causing an increase of GSH and its stabi-

lity as demonstrated in this paper. The elucidation of these supposition should await further investigation.

Another interest of the present case is genetic pattern. It has been mentioned that the enzymatic defect in erythrocytes of this type of hemolytic anemia may be transmitted as a sex-linked gene of intermediate dominance. The demonstration of enzyme deficiency in our patient, the presence of the same lesion to a lesser degree in her mother, and the absence of the defect in her father seems to agree with the genetic concept. Although a maternal cousin (a) was probably affected by the same lesion, it is possible to presume that the lesion was transmitted not from paternal side (A) but from maternal side (A') who came of the same kinship (Fig. 1). If this supposition is correct, the genetic pattern that the gene is carried by female might be accepted. It suggests a severer lesion because of hemizygote that the brother of the patient and her male cousin (a) died in early infancy. The deficiency of G6PD was milder in our patient as compared with Shahidi's cases, brothers. This difference might be explained by sex-difference, provided the abnormality is sex-linked. As seen from the pedigree of the present case (Cf. Fig. 1), the distribution of the affected gene is not always clear because of unaccuracy of statement by the parents. It is difficult to trace the genetic transmission without biochemical examinations, as a milder degree of enzyme defect and anemia were only disclosed by examinations in the mother who appeared to be normal. The manifestation of hemolysis may depend on the severity of gene penetrance on one hand and on the oppotunity such as infection or exposure to certain drugs which aggravate preexisting metabolic defect in the mildly affected case on the other hand.

SUMMARY

1) A case of congenital nonspherocytic hemolytic anemia was reported. A metabolic abnormality in the erythrocytes from the patient was demonstrated, consisting of a low content of reduced glutathione, an abnormality in the reduced glutathione stability test, and a low activity of glucose-6-phosphate dehydrogenase. These abnormalities were also found in the erythrocytes from the mother to a milder degree, while there was no abnormalities in the erythrocytes from the father. This is the first case in Japan, of this type of hemolytic anemia in which an enzymatic anomaly of the erythrocytes was demonstrated.

2) The treatment with large dose of vitamin B_{12} brought about a relief from jaundice, when an increase of reduced glutathione content in the erythrocytes and an improvement of reduced glutathione instability were observed, although the activity of glucose-6-phosphate dehydrogenase in the erythrocytes remained in a constantly low level.

3) The in-vitro effect of vitamin B_{12} on gulcose-6-phosphate dehydrogenase was investigated. The relationship between vitamin B_{12} and glutathione me-

tabolism was discussed.

4) Genetic mode of the disease was discussed.

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The Effects of Gonadotropin on Sex Steroids Production

By

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INTRODUCTION

It is generally known that gonadotropin secreted from the anterior lobe of the pituitary gland has direct or indirect influence upon the peripheral endocrine glands such as the ovarium, the testis and the adrenal cortex. However, the mechanism of the action of gonadotropin has not yet been made clear.

The most important biochemical reactions in a living body are generally considered to occur under the existence of some enzymes. The experiment to study the mechanism of secretion of sex hormones has recently been undertaken by elucidating a meaning of enzymatic reaction in the hormone production of a living body. However, the past reasonings about the mechanism of biosynthesis of sex hormones are no better than hypotheses, and many obscurities still remain unexplained.

We are making the experiments in order to elucidate such mechanisms. This time we are going to report the results up to this day about biochemical and electron-microscopic studies on the effects of gonadotropin on sex steroid production.

EXPERIMENTAL

I. The Effect of Gonadotropin on the Ovaries

1) Experimental Materials and Methods

a) Determination of estrogen in blood and urine:

2 adult females assured of normal endocrine functions by measurements of the basal body temperature and the estrogen contents in blood and urine were used as subjects. On the last day and the next day of menstruation, the estrogen values were measured. 5,000 IU of PMS (Anteron) dissolved in 500 ml of physiological saline was intravenously administered by instillation just after the second measurement. Thereafter, the estrogen values were measured on the first, the second, the fourth, the sixth and the twelfth days.

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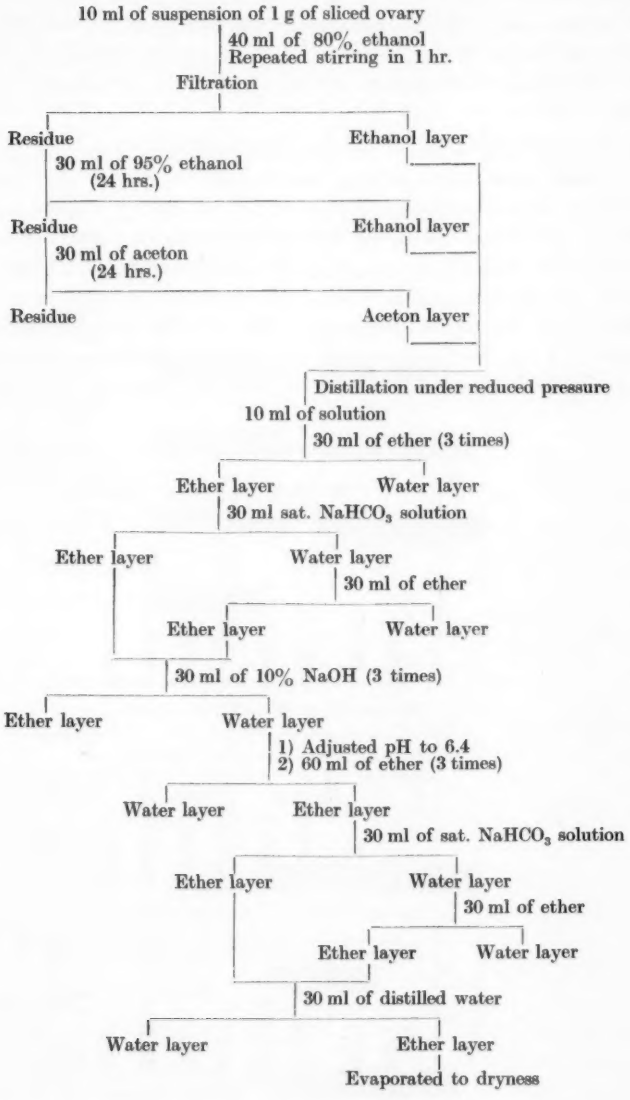
TABLE I. Determination of Estrogen from the Ovary



50 ml of 24 hrs. urine and 10 ml of blood plasma were sampled for each measurement. The crude estrogen was extracted with ether and NaOH solution after hydrolysis. The estrogen content was determined by fluorescence photometry after fractioning of the extract with elution column chromatography using mixture of methanol and benzene as the moving phase and alumina as the stationary phase[1),2),3)].

b) In vitro production of estrogen in the ovary:

Fresh adult swine ovaries were brought frozen to the laboratory. 10 ml of Krebs-Ringer phosphate solution of pH 7.4 and 1 g of sliced swine ovary were put in a Warburg bottle. A dosis of precursor was added and stirred at the speed of 60 rotations per minute at 37°C for 3 hrs. 200 IU of PMS (Anteron) was added as gonadotropin per test. Extraction of estrogen from the ovary was effected by the method tabulated in Tab. I. For purifying and fractioning the extract, it was first subjected to alumina column chromatography and then to paper chromatography according to Axerlord's method[4)] in principle.

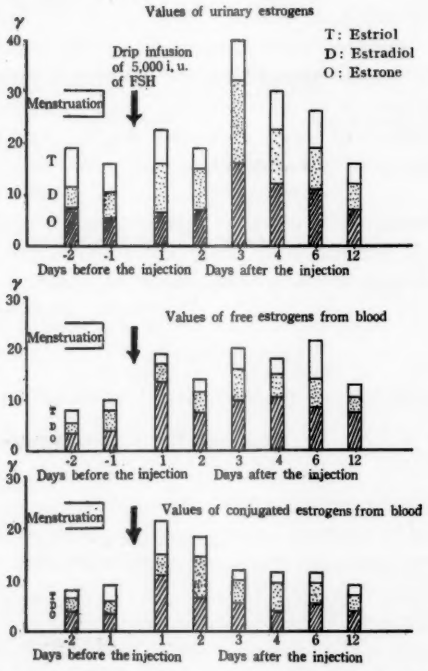


Fig. 1. Estrogen values from urine and blood before and after drip infusion of PMS.

2) Experimental Results

a) Increase of estrogen values of women following administration of gonadotropin: The mean value obtained with 2 normal subjects was as shown in Fig. 1. The blood estrogen values rose to the peak on the first day and the urine estrogen on the third day. Of the estrogen fractions, the increase of estradiol and estrone was particularly notable both in urinary and blood estrogen, indicating that administration of gonadotropin acts in increasing the sex hormone production in vivo.

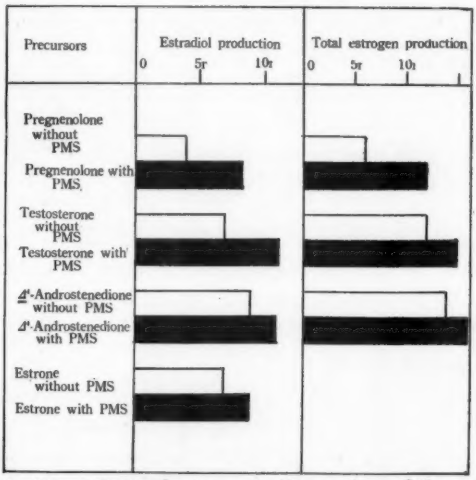


Fig. 2. Biosynthesis of estrogen in the ovarium of the swine.
r : gamma.

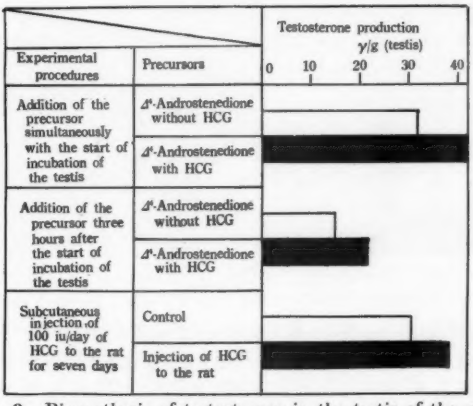


Fig. 3. Biosynthesis of testosterone in the testis of the rat.

b) Estrogen biosynthesis in ovaries:

The effect of gonadotropin on the production of estrogen in slices of swine ovary was as shown in Fig. 2. The values are given in the average value of 5 cases each. As precursor, 1,000γ of pregnenolone, testosterone or Δ^4-androstenedione or 40γ of estrone was used per test. Gonadotropin was found evidently effective in expediting the conversion of pregnenolone into estradiol and estriol, and showed effect in enhancing the production of estrogen following administration of testosterone, Δ^4-androstenedione and estrone as precursor.

II. Effect of Gonadotropin on the Testes

1) Experimental Materials and Methods

The testes extracted from adult male rats of Wistar-breed were immediately cut into slices for use in the following experiments. 10 ml of Krebs-Ringer phosphate solution and slices of one testis each were put in a Warburg's bottle, the precursor was added and incubated at 37°C for 3 hrs. under stirring. Then the testosterone content of these slices was measured.

For purification of testosterone, it was extracted by Slaunwhite-Samuels' method[5] processed by silica-gel column chromatography and subjected to paper chromatography by Savard's process and then by Bush A system. For quantitative determination, König's method[6] was adopted.

2) Experimental Results

The results were as shown in Fig. 3. The values are given in the mean values per series of measurements, which consisted of 5 rounds of measurements each. The production of testosterone was large when the sample was incubated with precursor and the testis fresh after the sampling. But the testosterone production was lowered, when the precursor was added 3 hrs. after the beginning of incubation of the sample, and then the sample was incubated for 3 hrs. When 500 IU HCG (Premogonil) was added per incubating tube, the testosterone production upon incubation was found enhanced.

III. Observation of Ovaries under Electron Microscope

1) Experimental Materials and Methods

The ovaries of Wistar-breed adult rats of 150–250 g of body-weight were extracted. The granulosa cell and the theca cell of the follicle of the ovaries were immediately fixed in a mixed solution of 2 % osmic acid and veronal-acetic acid buffer[7),8], in an ice-box for 3 hrs. The osmic acid was removed from the fixing solution by washing thrice with this liquid. Then the sample was dehydrated in 70%, 80%, 90%, 95%, 100% and 100% ethanol in succession for 30 min. each or for 3 hrs. in total. For embedding agents, a mixture of 80% of N-butylmetacrylate and 20% of methylmetacrylate mixed with 2% of 2,4-dichlorobenzolperoxide as polymerization promoter was used. Polymerization was allowed to

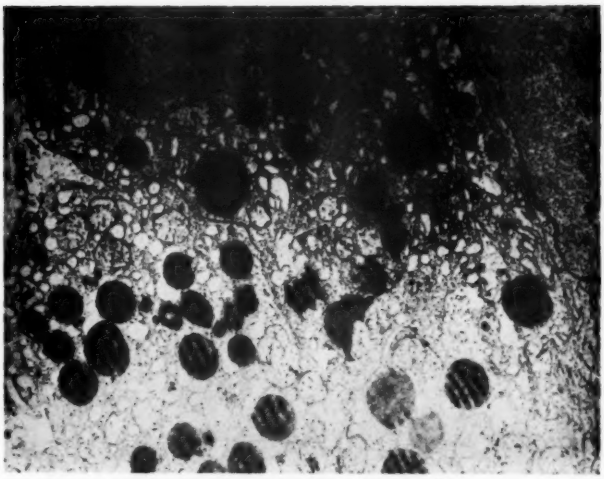


Fig. 4. Fine structure of theca cells.

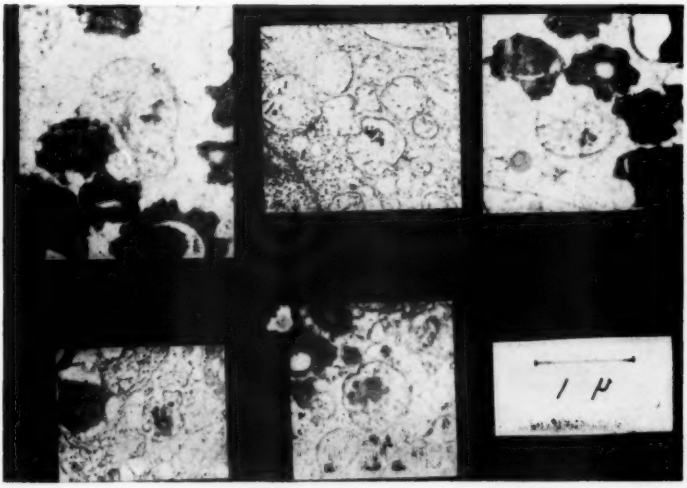


Fig. 5. As in Fig. 4, further explanation in text.

go on for 6 hrs. at 53°C. The embedded specimens were then cut by a Nipponkogaku Work's JUM4 ultramicrotome into sections of ca. 0.05 μ. A Hitachi Work's HU 10 type electron microscope was employed at the direct magnification of 2,000–10,000, without removing the embedding agents.

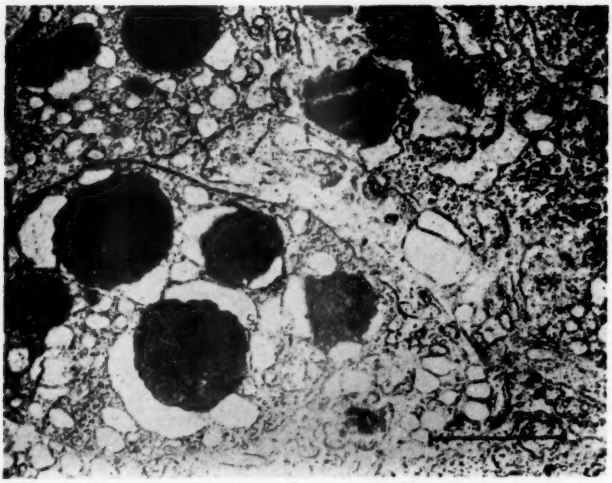


Fig. 6. Smooth-surfaced endoplasmic reticulums in theca cells.

2) Experimental Results

In Fig. 4. is shown the fine structure of the theca cell of a mature follicle. We find numerous granules apparently of fat. Mitochondria with somewhat changed inner-structure, cristae with changed arrangement and fat granules invading the mitochondria are visible in some cases as shown in Fig. 5. Steroid hormone is fat-soluble and is found in fat of the living body. An oxidation-reduction reaction comes into the process of elaborating the steroid hormone. The mitochondria contains many sorts of oxidase-deoxidase enzymes. Therefore, it may be inferred that the mitochondria is related in some way with the estrogen-production process.

Fig. 6. shows smooth-surfaced endoplasmic reticulums in theca cells of a mature follicle. It contains imported fat granules, presumably correlated with release of sex hormone.

DISCUSSION

FSH released from the hypophysis causes growth of the granulosa cells of the follicle of the ovary, then a small quantity of LH comes in and estrogen is secreted increasingly from the matured follicles. This is followed by an abrupt increase in the secretion of LH, up-setting the equilibrium between the quantities of FSH and LH, and ovulation follows. Such has been the generally accepted notion on the hormone secretion in one sexual cycle[9),10)]. But nothing has been blamelessly demonstrated on what kind of biochemical reaction gonadotropin causes in the ovary.

We found that production of estradiol from pregnenolone was increased by gonadotropin at the level of significance of 5 %, and that from testosterone and estrone at the level of significance of 1%. It seems that the mechanism of action of gonadotropin in the process of estrogen production is as follows: 1) Gonadotropin accelerates the production of benzoic A-ring of estratriene in the ovary. The principal unit reactions here are expedited by desmolase causing disbonding of C_{10}–C_{19} and by hydro- as well as by dehydrogenase centering on the transposition of H atoms. 2) Gonadotropin may expedite the reaction concerning in 17-hydrogenase of the ovary, but not in that of dehydrogenase, for it accelerates elaboration of estradiol from estrone, but not vice versa.

The details on the action of gonadotropin on the function of testosterone secretion are yet unknown, as its action on the ovary. At present, it is generally accepted that testosterone in the testes is produced via the process of acetate→cholesterol→pregnenolone→progesterone→Δ^4-androstenedione→testosterone.[11)–14)] Brady[15)] (1951) found that addition of 2 mg (6,400 IU) of chorionic gonadotropin promoted the production of testosterone to 2½–10 folds in the rabbit testes incubated in acetate-C^{14} solution; but seeing that the cholesterol content was not affected at all in the process, he opined that cholesterol does not come into the biosynthetic process. Savard *et al.*[11)] (1952) also found addition of hypophyseal gonadotropin caused increase of the neutral steroid fractions to more than twice, in their experiment with human testes perfused with acetate-C^{14}. Helmreich[16)] and Samuels[17)] made observations on the action of gonadotropin during the process of pregnenolone→ progesterone and the former concludes that gonadotropin assists the enzymal activity only secondarily, by expediting the synthesis of protein in the stromal cells, instead of directly activating the enzymal action. Brink-Johnsen[18)], in their experiments with perfused canine testes, isolated testosterone and Δ^4-androstenedione from blood in the seminal vein. They found the rapid increment of secretion of androgen into the seminal vein blood after administration of human chorionic gonadotropin. This finidng suggests that human chorionic gonadotropin either causes release of hormone existing in the testes or promotes the biosynthesis of hormones. He says that the latter deduction seems to be the more rational, seeing that the testosterone content in the testes is very small. He says also that gonadotropin may act on the hormone production either by directly enhancing the activity of the existing enzymes or by promoting the elaboration of such enzymes from the precursory substances, but the former seems to be the more reasonable conclusion, since the time required for HCG to enhance the androgen secretion is short.

The present authors have seen that HCG acts in promoting the Δ^4-androstenedione → testosterone reaction, but this action is effective in promoting the synthetic process by only 30–40% over that in normal controls. Takagi[19)], in his experiments of incubating specimens of human testes, found a rather large

number of fibroblasts growing and proliferating in the form of stout cords. After all, the question of how gonadotropin acts in the series of biochemical reactions by which androgen is synthesized remains as yet in the dark.

SUMMARY

We studied the effects of gonadotropin on the biosynthesis of sex hormones in the sex glands and it was shown that gonadotropin accelerates the biochemical reactions producing sex steroids in the sex glands.

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A New Simple Screening Test for Pheochromocytoma

By

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INTRODUCTION

It is very important in clinical practice to diagnose pheochromocytoma, because this disease is surgically curable one. Although the diagnosis of this disease is currently made by some pharmacological tests or by the determination of catecholamines in urine or in blood, the former not infrequently gives false-positive or negative results and the latter is not popular because of its technical difficulties and of need of special equipments.

Therefore, it is desirable to develop a simple, reliable and fairly rapid test to diagnose this disease.

On the other hand, it has been shown recently that both adrenaline and noradrenaline are metabolized to 3-methoxy-4-hydroxymandelic acid (Vanillylmandelic acid, VMA) as their end product[1]. In addition, 3-methylated derivatives of catecholamines, metadrenaline (MA) and normetadrenaline (NMA) have been demonstrated in urine from patients with pheochromocytoma[2]. These studies suggest that the analysis of such compounds in urine will lead to the diagnosis of this disease.

But the determination of these compounds cannot be easily applied for screening purpose because it requires considerable time and specialized techniques.

When these compounds are plotted on filter paper, a violet color is developed by spraying diazotized p-nitroaniline on it, and a solution of these compounds in test tube shows a wine color with addition of this reagent.

We employed this characteristic color reaction of these compounds and developed a new simple screening method for the detection of pheochromocytoma.

EXPERIMENT

(A) Reagent: Diazotized p-nitroaniline was used as reagent, as proposed by Studnitz *et al.*[3]

佐藤辰男，吉永　馨，石田　望，伊藤忠一，和田保男

(1) 0.1 g. of p-nitroaniline dissolved in 2 ml. of conc. HCl and diluted with water to a volume of 100 ml.

(2) 0.2 % sodium nitrite.

(3) 10 % potassium carbonate.

All these solutions were stored at 4°C. Just before use, one volume of (1) and one volume of (2) were mixed. To this mixture was added two volumes of (3). This mixture must be used within 120 seconds.

(B) Experimental procedure: There are two types of our method, one of them is named one spot method and the other test tube method.

(1) One spot method: Using a small pipette, raw urine sample is plotted on filter paper to make a spot of about 0.5 cm. After drying at room temperature, the paper is sprayed with the mixed reagent mentioned above, to test the color of the spot developed on the paper.

(2) Test tube method: To about 1 ml. of raw urine is added the same volume of the mixed reagent, to test the color tone in test tube.

RESULTS

In both methods, color reaction takes place just after spraying or adding of the reagent.

(1) One spot method: In the urine samples from normal subjects and patients without pheochromocytoma, there appeared only faintly yellow or orange part at the center of the spot and a light red part in the periphery (negative reaction), but the urine spots from two patients with pheochromocytoma showed distinct and diffuse violet coloring (positive reaction).

VMA content in urine from these patients with pheochromocytoma was determined by high voltage paper electrophoresis, then this urine was diluted gradually in order to determine the lowest content of VMA in urine to give positive reaction. From this experiment, it was found that a urine sample containing more than 10 μg VMA/ml. showed distinct violet color, and urine samples containing 6–8 μg VMA/ml. could be distinguished from normal urine samples. This color reaction was not affected by the time interval, at least up to 48 hours elasped from the plotting of urine to the spraying of the reagent.

(2) Test tube method: Urine samples from patients with pheochromocytoma showed distinct wine color, while urine samples from normal subjects or other patients only yellowish brown. In this method, the lowest content of VMA giving positive result was 6–8 μg/ml. No violet or wine color was observed in glycosuria and albuminuria from patients without this tumor. In addition, the color reaction of urine stored at 4°C and room temperature was examined repeatedly during 7 days, the same results were obtained.

DISCUSSION

It is well known the clinical symptoms of pheochromocytoma are hypertension, tachycardia, sweating, increased basal metabolic rate, glycosuria, albuminuria etc. which result from excess of catecholamines released from the tumor in the adrenal medulla or paraganglia, and that all these symptoms disappear postoperatively. Therefore, its correct diagnosis is very important in practice.

TABLE I. Urinary Excretion of VMA Reported by Various Authors (mg/24 hours)

	Normal Subjects	Patients with Essential Hypertension	Patients with Pheochromocytoma
Armstrong *et al.*[4]	2.0~4.0 (?)		9 ~90* (8)
Robinson *et al.*[5]	(30) 1.3~3.6	(100)	10.8~15.8 (4)
Sandler *et al.*[6]	11.0±2.3 (11)		19.0~60.5 (4)
Kraupp *et al.*[7]	(4) 0.5±0.16	(4)	9.5~17.0 (1)
Sunderman *et al.*[8]	3.9±1.6 (30)		15 ~250 (5)
Gitlow *et al.*[9]	1.4±0.38* (15)	1.4 ±0.6 * (36)	6.0~40.0* (30)
Studnitz[10]	4.84±0.10 (67)	5.64±0.15 (105)	6.6~380 (13)
The authors	3.15±0.73 (24)	3.14±0.79 (42)	9.6~54.0 (2)

( ) number of cases. * in μg/mg creatinine.

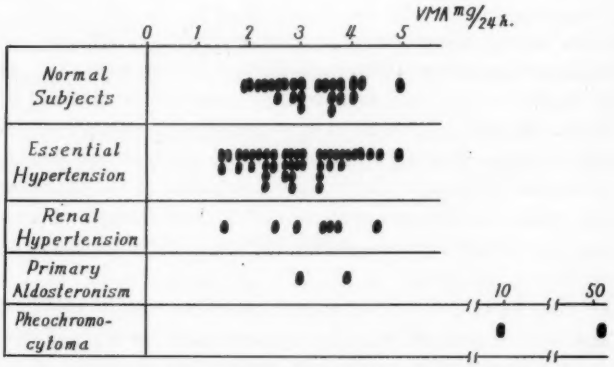

Fig. 1. Urinary excretion of VMA in normal subjects and patients with hypertention.

In 1957, Armstrong *et al.*[1] demonstrated VMA in human urine as the final metabolic product of catecholamines. And they also found that this substance is excreted in abnormally large amounts in urine from patient with pheochromocytoma. Soon afterward, LaBrosse[2] reported that large amounts of MA and NMA are excreted in such cases. These investigations suggested that

the diagnosis of pheochromocytoma could be made by analysis of urine for these metabolites of catecholamines.

Tab. I. illustrates the values of urinary VMA excretion which were reported so far by several authors. These results afford differentiation of normal subjects or patients with essential hypertension from those with pheochromocytoma in every instance. Our data showed good agreement with these results. In addition, it was found by our investigation that patients with renal hypertension and primary aldosteronism excrete equal amount of VMA in their urine as compared with normal subjects (Fig. 1.).

TABLE II. Urinary Catecholamines and Their Metabolites in Patients with Pheochromocytoma

	Case 1.	Case 2.
Adrenaline*	3510 μg/24 hrs.	240 μg/24 hrs.
Noradrenaline*	4340 〃	616 〃
Metadrenaline†	31.4 mg/24 hrs.	7.7 mg/24 hrs.
Normetadrenaline†	5.6 〃	8.3 〃
Vanillylmandelic acid‡‡	54.0 〃	9.6 〃

* Euler & Folding's method.
† High voltage paper electrophoresis (Yoshinaga *et al.*[11])
‡‡ 〃 〃 (Studnitz *et al.*[3])

There are several procedures for the determination of VMA in urine, for example, two-dimensional paper chromatography by Armstrong *et al.*[12], colorimetric method by Sandler *et al.*[6] and high voltage paper electrophoresis by Studnitz *et al.*[3]. These procedures have to be employed for the separation of VMA from other phenolic compounds and for the quantitative study of this substance. Tab. II. lists the results in the analysis of these metabolites of catecholamines in urine from our two cases of pheochromocytoma. All of these methods of determination, however, are difficult and time-consuming, and are ill suited for the routine clinical laboratory.

On the other hand, our method is very simple and can be readily carried out without any use of specialized and expensive equipments. Furthermore, it needs very little time, so that many urine samples can be treated at a time.

Armstrong *et al.*[12] reported that there are about 40 phenolic acids besides VMA in urine and 10~17 compounds of them are always found by his chromatographic separation. Thus, it seems somewhat questionable to use raw urine itself in our procedure. However, it may be emphasized that our method is only a qualitative test to see whether the urine contains as much amount of VMA, MA and NMA as the urine from patient with pheochromocytoma.

The one spot method may be useful for mass examination for hypertensives,

and the test tube method may be convenient for routine laboratory use. In addition, the sensitivity of these methods is fairly high, the test gives always positive result when urine contains more than 10 μg/ml. If urine contains a small amount of VMA of 6~8 μg/ml., positivity may be established by comparison with normal control urine. According to our data, normal urinary excretion of VMA, MA and NMA are ranging 1.4~4.5 μg., 0.05~0.2 μg., 0.05~0.5 μg/ml. respectively.

It is known, however, that severe physiological or mental stress, congestive heart failure, burn, shock, insulin administration etc. may result in an increased production of catecholamines and thus may be associated with abnormally high excretion of their metabolites. Therefore, our method may lead to positive result in these circumstances. But this possibility does not limit the usefulness of our method, since the clinical symptoms in these diseased states may easily expose the false-positive result. Moreover, false-negative results may occur by our method in patients with pheochromocytoma who excrete only a small amount of these metabolites. As shown in Tab. I., several cases of pheochromocytoma excreting relatively small amount of VMA in their urine were reported by Gitlow *et al.*[9] and Studnitz[10]. From these reasons, caution must be exercised in using our method. We have experienced only two cases of pheochromocytoma to date, thus the accuracy of this method should be established by further investigation over many cases of this disease.

Recently, several screening tests based on VMA estimation have been reported by Gitlow *et al.*[13] and Robinson *et al.*[5] But the former needs extraction procedure of urine and employment of spectrophotometer, and the latter is much time-consuming. Thus many urine samples cannot be treated in short time by these methods.

From these reasons, our method might be the most suitable one, and hypertensive subjects can be quickly and easily screened for the presence of pheochromocytoma.

SUMMARY

(1) A very rapid and simple method for screening pheochromocytoma was described.

(2) This method was based on a characteristic color reaction of the catecholamine metabolites, VMA, MA and NMA, to diazotized p-nitroaniline.

We are grateful to Dr. M. Armstrong for gifts of VMA, to Prof. S. Seto and Dr. Y. Ikegami, Chemical Research Institute of Non-aqueous Solution, Tohoku University, for the synthesis of VMA at our request and to Dr. J. Axelrod for giving us MA and NMA.

We are indebted to Prof. Dr. T. Torikai for his continuous leadership throughout this study.

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Experimental Studies of the Electrocardiographic Effect of Potassium Solution on a Toad's Heart in the Presence of Insulin and Glucose

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INTRODUCTION

In a previous report on the electrocardiographic effect of low potassium solution on the damaged area of the heart surface, we noted the following results[1].

Immediately after injury, the ST-elevation was, of course, recorded in the direct unipolar electrocardiogram taken from the injured heart surface. This elevation of the ST-segment was not eliminated even when potassium-free solution was poured on the injured region. As time passed, however, it did not make the ST-elevation whereas the normal Ringer's solution did. Thereafter it was necessary to use samples of higher concentration to produce the ST-elevation as the course progressed; the concentration of potassium solution was higher to make the ST-elevation. In other words, there exsists a kind of threshold of potassium concentration to produce the ST-elevation and its value rises as time passes after injury. These findings are explained by the supposition that the intracellular potassium was gradually restored after injury, so that the higher concentration was needed to produce the ST-elevation.

As the course of the change in the ST-deviation progressed, there came a transient phenomenon, that in order to produce the ST-elevation a higher concentration of potassium was needed than the normal threshold level seen in an intact heart; the threshold level of potassium was temporarily higher than normal at the end of recovery. It is, therefore, considered that the injured heart cell may take up potassium more than normal at recovery. That the ratio of intra- to extracellular potassium concentration exerts an influence upon the ST-deviation, was shown in the previous experiment.

Then, if the intracellular potassium is possible to increase, the ratio of intra- to extracellular potassium must be altered. When the heart, in which the

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intracellular potassium increases more than normal, is covered with potassium solution, an altered ratio will occur between intra- and extracellular potassium. It is reasonably thought that such an alteration of the ratio may have an effect on the ST-elevation, as observed in the case of the decreased myocardial potassium.

Choosing insulin as a way of increasing the intracellular potassium, we at first examined the effect of insulin on the myocardial potassium and secondly studied the electrocardiographic effect of potassium solution on the heart treated with insulin and glucose.

EXPERIMENTAL METHOD

Two kinds of experiments were performed. In the first, a toad's heart was isolated carefully from the body, so as not to be injured. Blood was perfectly excluded. The heart was put into the Ringer's solution of a certain volume which contained 100 mg per dl of glucose and 5 units per dl of insulin, and in which the potassium concentration was 7 or 4 mEq/L (Fig. 1). At 30 minutes,

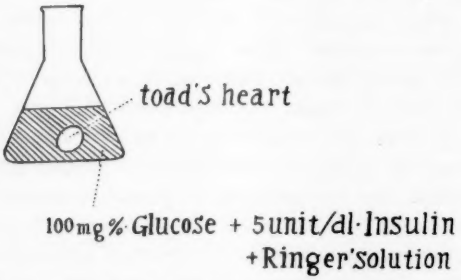

Fig. 1. Experimental method (1). Potassium and sodium contents of the solution were determined with time by use of a flame-photometer.

60 minutes, 120 minutes, 150 minutes, and 180 minutes after the beginning of the experiment, potassium and sodium contents of the solution were determined with a flame photometer. Temperature of the solution was held at 15–17 degrees centigrade. After the determination, the heart was blotted gently by a dry filter-paper to remove the solution and tissue fluid, and the weight was measured on torsion balance.

The second experiment was performed as follows. Ten toads were used. Wide exposure was accomplished by removing the frontal chest wall. The pericardium was cut open carefully, so as not to injure the heart surface. Then the heart was poured over continuously by the above-mentioned insulin-glucose-Ringer's solution. Meanwhile, the heart surface was often applied with the

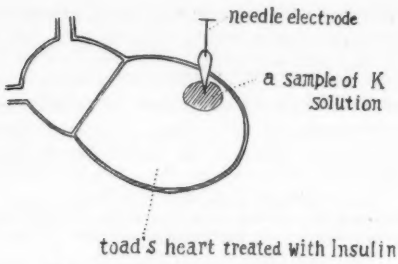

Fig. 2. Experimental method (11).

previously prepared samples of the potassium solutions and the direct unipolar leads were taken through them. A needle electrode wrapped with moist cotton was employed as the different one (Fig. 2). The indifferent electrode was placed on the right arm. The elevation of the ST-junction was used as a criterion of the deviation of the ST-segment. The samples containing the same constitutents as Ringer's solution except for potassium were prepared, in which the potassium concentration was altered from 4 to 10 mEq/L.

RESULTS

1) Changes in the potassium and sodium concentrations of the insulin-glucose-Ringer's solution after immersion of the heart.

Potassium content of the insulin-glucose-Ringer's solution was decreased with time after immersion of the heart, as shown in Fig. 3. This graph indicates that the isolated heart took up potassium from the solution in the presence of

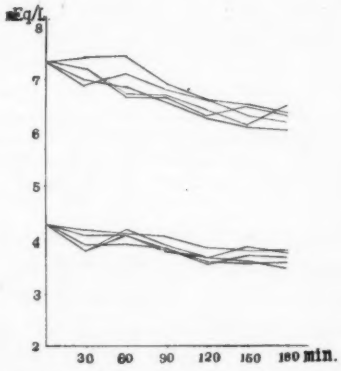

Fig. 3. Changes in the potassium concentration of the solution. The upper group shows the cases of 7 mEq/L potassium in the insulin-glucose-Ringer's solution, and the lower, 4 mEq/L potassium in it.

TABLE I. The myocardial potassium of a toad is regarded as 109.2 mEq per kg of fresh tissue, and the potassium of each heart is estimated after the experiment. Potassium entering heart tissue is calculated from the difference of potassium concentrations before and after the experiment. It is shown by the bracket number in this table. Nos. 1–5 belong to the cases of 4 mEp/L potassium in the insulin-glucose-Ringer's solution and Nos. 6–10 are the cases of 7 mEq/L potassium in it. Details in text.

Case	Weight of heart	Estimated heart K	K entering heart
No. 1	907 mg	99.0 μEq	0.76 mEq/L (3.8 μEq)
No. 2	315	34.4	0.72 (3.6)
No. 3	433	47.3	0.47 (2.4)
No. 4	418	45.6	0.61 (3.1)
No. 5	499	54.5	0.85 (4.3)
No. 6	533	58.2	0.90 (4.5)
No. 7	512	55.9	1.10 (5.5)
No. 8	335	36.6	0.85 (4.3)
No. 9	430	46.9	1.04 (5.2)
No. 10	315	34.4	0.97 (4.9)

insulin and glucose. It also shows that the heart in the insulin-glucose-Ringer's solution with 7 mEq/L potassium in it took up more potassium than the heart in the solution with 4 mEq/L potassium. Potassium change was calculated from the difference in the concentrations before and after the experiment. In the cases of the insulin-glucose-Ringer's solution containing 4 mEq/L potassium, the potassium change after experiment was calculated as 2.4 to 4.3 micro Eq (3.4 micro Eq on the average), and in the cases of 7 mEq/L, 4.3 to 5.5 micro Eq (4.9 micro Eq on the average) was obtained (Tab. I).

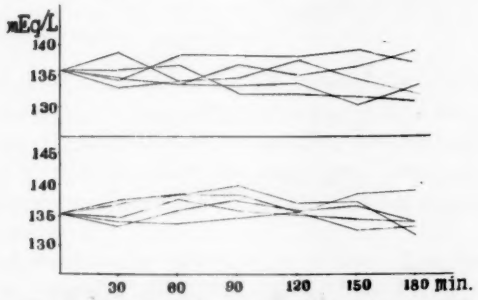

Fig. 4. Changes in the sodium concentration of the solution. No uniform change is observed. The upper group shows the cases of 7 mEq/L potassium in the insulin-glucose-Ringer's solution and the lower belongs to the cases of 4mEq/L potassium in it.

As to sodium, no uniform changes were observed, as shown in Fig. 4.

These facts suggest that potassium entered the heart tissue and the intracellular potassium was consequently increased. From this viewpoint, the insulin-glucose-Ringer's solution was considered to be usable for the present study, in which the intracellular potassium is desired to be increased in the heart, and will be discussed later.

2) The electrocardiographic effect of potassium solution on the heart treated with the insulin-glucose-Ringer's solution.

Immediately after the insulin-glucose-Ringer's solution was administrated to the epicardial surface, 6–7 mEq/L potassium solution obtained the ST-elevation. As time passed and the action of the insulin-glucose-Ringer's solution continued, however, came the moment when the ST-elevation was no longer recorded if 6-7 mEq/L solution was used. Thereafter it was necessary to use samples of higher potassium concentration in order to generate the ST-elevation.

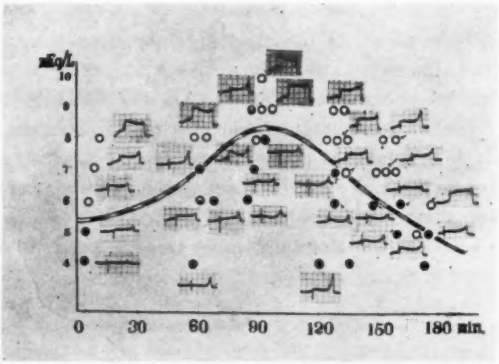

Fig. 5. A course of the change in the ST-deviation after the administration of the insulin-glucose-Ringer's solution to the epicardial surface. The surface was covered with potassium solutions of various concentrations and the direct unipolar electrocardiogram was taken from there. The white spots indicate the ST-elevation, the black ones no deviation.

Fig. 5. shows an instance of the course of experiment. Before and soon after the administration of the insulin-glucose-Ringer's solution, the ST-elevation was recorded by 5–7 mEq/L potassium. As 60 minutes passed, 6–7 mEq/L solution did not produce the ST-elevation, whereas 8 mEq/L had the effect. At 90 minutes the ST-elevation was not obtained by 8 mEq/L, but 9 or 10 mEq/L solution produced the ST-elevation. At that moment, the pouring of the insulin-glucose-Ringer's solution was ceased.

At 130 minutes from the beginning, 6–7 mEq/L solution had no effect on

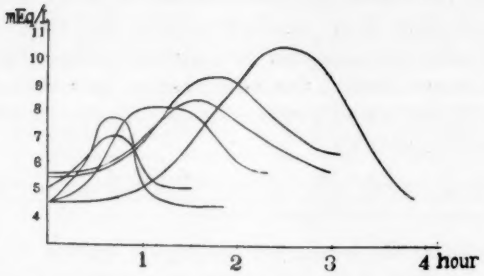

Fig. 6. Boundary curves obtained in each experiment. Cases of 4 mEq/L potassium in the insulin-glucose-Ringer's solution.

the ST-segment, but 8 mEq/L produced the ST-elevation. As time continued to pass, the ST-elevation was obtained by 7 mEq/L and at 175 minutes 6 mEq/L solution, again.

In this figure the white spots represent the ST-elevation and the black ones are used for no ST-elevation. A boundary curve between these white and black spots may be drawn as seen in the figure. Such curves were obtained in each experiment performed in six cases (Fig. 6). On the top side of the curve the ST-elevation is recorded, while in the lower part no ST-elevation is found. This fact shows that there existsts a kind of threshold of potassium concentration to generate the ST-elevation and its value rises as time passes after the administration of the insulin-gulcose-Ringer's solution. After the cessation of the insulin-glucose-Ringer's solution, the threshold value was observed to return gradually to the previous level of 6–7 mEq/L.

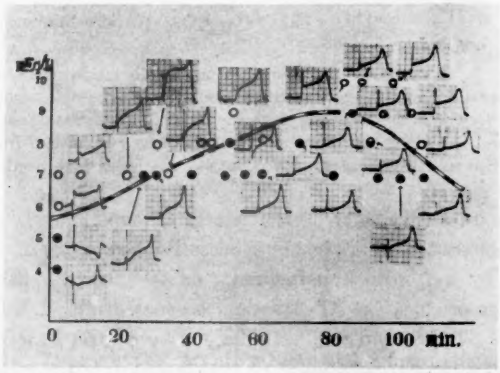

Fig. 7. A course of the change in the ST-deviation after the administration of insulin-glucose-Ringer's solution to the epicardial surface. The solution contained 7 mEq/L potassium. Details in text.

Fig. 7. shows another case of the experiment. In this case, the insulin-glucose-Ringer's solution containing 7 mEq/L was used. Immediately after the administration of the insulin-glucose-Ringer's solution, the ST-elevation appeared as when the 7 mEq/L potassium was used itself. 30 minutes after the experiment was begun, however, the ST-elevation was not recorded by 7 mEq/L solution, whereas 8 mEq/L had the effect. After this, the ST-elevation was not recorded as long as 7 mEq/L solution was used. At 50 minutes 8 mEq/L had no effect on the ST-segment, whereas 9 mEq/L had an effect. At 85 minutes the ST-elevation was obtained by 10 mEq/L, and not by 9 mEq/L.

At this time, the administration of the insulin-solution was discontinued. During the next 30 minutes after cessation, came the moment when 9 mEq/L

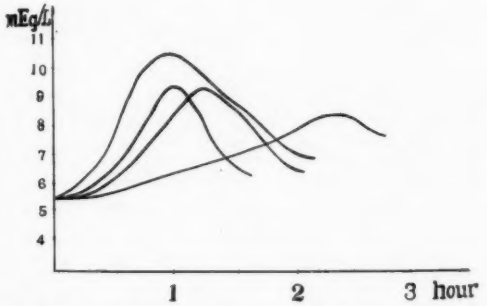

Fig. 8. Same as in Fig. 6. Cases of 7 mEq/L potassium in the insulin-glucose-Ringer's solution.

Table II. Nos. 11–16 are the Cases of 4 mEq/L Potassium in the Insulin-glucose-Ringer's Solution, and Nos. 17–20 Belong to the Cases of 7 mEq/L Potassium in It. Refer to Figs. 6 and 8. Details in text.

Case	Time-course of elevated threshold level	The highest threshold of K conc. in course	ST-depression noted by normal Ringer's solution
No. 11	30 min.	7-8 mEq/L	+
No. 12	120	9	+
No. 13	80	8	+
No. 14	110	10	+
No. 15	40	7	−
No. 16	60	8-9	−
No. 17	75 and more	8-9	+
No. 18	75	9-10	Ringer's not used
No. 19	50	9-10	Ringer's not used
No. 20	90 and more	10 and more	+

of 8 mEq/L potassium solution was able to produce the ST-elevation again. In this case too, it was observed that the threshold value to produce the ST-elevation rose as time passed after the administration of the insulin-glucose-Ringer's solution and returned gradually to the previous level after the cessation. The same results were obtained in four cases of 7 mEq/L potassium in the insulin-solution, as seen in Fig. 8.

It was observed that the time-course of the elevated value of potassium to produce the ST-elevation was different in different cases. It varied from 30 to 120 minutes. The highest threshold of potassium in each case was also different, but it seems to have been higher in the cases of 7 mEq/L potassium in

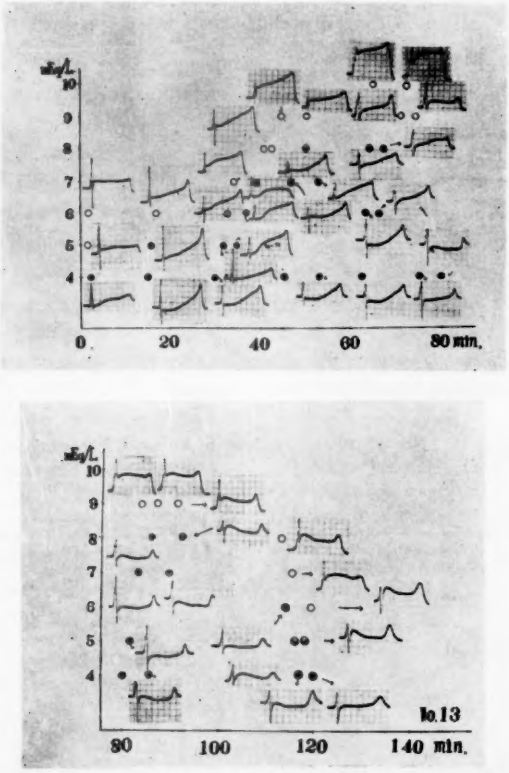

Fig. 9. The change in the threshold value in a case of 4 mEq/L potassium in the insulin-glucose-Ringer's solution. When the threshold value is elevated to 10 mEq/L for instance, the Ringer's solution containing 4 or 5 mEq/L potassium produces the ST-depression instead of the elevation.

the insulin-glucose-Ringer's solution than in the cases of 4 mEq/L potassium (Tab. II).

When the threshold value rose to 10 mEq/L for instance, the ST-depression was noted as soon as the normal Ringer's solution containing 4 or 5 mEq/L potassium was used. After the threshold value returned to the previous normal level, the normal Ringer's solution did no longer produce the ST-depression. Fig. 9 shows an instance of the relation. This electrocardiographic finding was seen in six cases out of ten. In the two remaining cases, the normal Ringer's solution was not used when the threshold value rose higher.

DISCUSSION

1) The change in the ratio of intra- to extracellular electrolytes by insulin.

We[1] have found that the potassium solution of low or normal concentration produces the ST-elevation if the intracellular potassium is decreased. From this observation it is considered that the intracellular potassium content has an effect on the electrocardiogram. It is, therefore, supposed that a change may occur in the electrocardiogram when the intracellular potassium is increased, but there exists little study related to the problem. From this standpoint, we have performed the experiments to see what change occurs in the ratio between intra- and extracellular potassium by insulin, as described previously.

At present, potassium salts, acetylcholine, some kind of corticosteroid hormone, digitalis, and insulin are cited as drugs that make the intracellular potassium increase. Among these, potassium salts are reasonably unsatisfactory to use, because the purpose of this experiment is to study the electrocardiographic effect of the potassium solution itself. As known so well, the increment of tissue glycogen is accompanied with the increase of potassium in tissue, and on the one hand it is reported that the intracellular glycogen is increased in the heart by acetylcholine[2]. The intracellular potassium is expected to be increased by acetylcholine. But Harris and Hutter[3] have recently observed in the experiment with radioactive potassium that potassium migration occurred in both directions by the action of acetylcholine from extra- to intracellular space and vice versa in the heart muscle of the toad and tortoise. Holland *et al.*[4] also reported similar results in the rabbit atria. Then it is unfit to use acetylcholine, in order to expect a unilateral increase of the intracellular potassium. Flückiger and Verzàr[5] observed that both glycogen and potassium were increased in a rat diaphragm immersed in hydrocortisone solution, but few experimental observations like this have been done, so is not generally accepted. In congestive heart failure clinically, digitalis is said to restore the intracellular potassium in the heart[6], but it is questionable whether the intracellular potassium is increased above normal, and it is reported experimentally that digitalis makes the intracellular potassium decrease in the heart of animals[7),8),9]. So it is

useless to apply digitalis for the purpose of making intracellular potassium increase.

On the other hand, Greenman[10], Fenn[11], Hastings *et al.*[12], and Teng *et al.*[13] reported that insulin causes the increase of glycogen and potassium in the muscle and liver as well as reducing blood sugar and serum potassium. Kamminga and collaborators[14] observed that an isolated rat diaphragm immersed in the insulin-glucose-Ringer's solution took up potassium from the surrounding area during the first hour of incubation. Yoshitoshi[15] reported the same relations in the heart of a dog. From these views, the authors applied insulin as a way of increasing the intracellular potassium in a toad's heart.

Now in the prescription of the insulin-solution, the concentration of insulin was decided to be 5 units per dl following Manery *et al.*[16] who studied the potassium absorption of a toad muscle by insulin. We added glucose to the insulin-solution because there is a well known affinity between the glucose and potassium uptake by the cell in which glucose does not enter the cell independent of potassium. As to the concentration of glucose in the insulin-solution, Kaminga *et al.* put 200 mg per dl of glucose into the solution in which the rat diaphragm was immersed although in the rat the blood sugar content is 120 to 140 mg per dl, and for the toad it is 45 to 65 mg per dl. In our experiment the concentration of glucose in the insulin solution was 100 mg per dl. Because 4 mEq/L potassium solution does not produce the ST-elevation, but 7 mEq/L has definitely a different effect electrocardiographically, potassium concentration was determined as 4 or 7 mEq/L in the insulin-glucose-Ringer's solution.

Because of the above-mentioned reasons, we performed the experiment to see the potassium migration into the heart muscle by action of insulin. When a toad's heart was immersed into the insulin-glucose-Ringer's solution, the potassium content as time passed decreased in the solution. This fact indicates that the isolated toad's heart took up potassium from the surrounding solution, and it was considered that by the action of insulin the intracellular potassium increased in the heart.

Relating to the problem to what degree the intracellular potassium can increase, there have been only a few reports. It is because there may be physiologically no condition of the increasing intracellular potassium above normal. Crismon *et al.*[17] found that the heart muscle potassium was raised significantly by the intraperitoneal administration of potassium; the normal heart muscle contains about 40.1 mEq per 100 g of fat-free dry tissue, while it increases to 43–54 mEq after administration. Ernst[18] observed in experiments with radioactive potassium that the myocardial potassium increased by 10% on the average in the isolated frog heart perfused with potassium-rich Ringer's solution.

In our experiments, the intracellular potassium was not directly determined.

It was estimated indirectly from the weight of the heart. According to Jojima and Kuriyama[19] potassium in the ventricle of a toad's heart is 99.5 mEq per kg of fresh tissue, excluding extracellular fluid and in the auricle 118.9 mEq per kg. The mean value of the both was adopted by us as the myocardial potassium of a toad. Absorbed potassium by an isolated toad's heart was 4.3 to 5.5 micro Eq (4.9 micro Eq on the average) in the insulin-glucose-Ringer's solution with 7 mEq/L potassium and it showed 2.4 to 4.3 micro Eq (3.4 micro Eq on the average) in the solution containing 4 mEq/L potassium. So the increase of the myocardial potassium is supposed to be about 10.5% (7.7 to 14.2%) in the former case and 6.0% (3.8 to 10.4%) in the latter. Tab. II shows the relation.

These results show that insulin makes the intracellular potassium increase and at the same time the surrounding solution of the higher potassium content causes the heart to take up more potassium than the lower potassium solution does. It is, therefore, possible that the intracellular potassium is increased, the extracellular potassium decreased, and consequently the ratio of intra- to extracellular potassium is altered by the administration of insulin.

Next, the effect of sodium must be taken into consideration. Yoshitoshi showed that sodium decreases in the myocardium after the injection of insulin. In experiments with radioactive sodium Flückiger and Versàr[20] pointed out that the presence of insulin and glucose in Ringer's solution gave a lower sodium content in the muscles as compared with controls in which both were absent. In the intracellular electrocardiogram, as Weidmann and others[21] reported, the membrane potential varies with the concentration ratios of sodium and potassium in extra- and intracellular fluids. If a change occurs in the myocardial sodium by administration of insulin, the membrane potential may be changed. In this regard, Creese *et al.*[22] and Zierler[23] showed that a decrease of the myocardial sodium and a change of the membrane potential are accompanied with the administration of insulin. Yamamoto[24] observed that the potassium-rich Ringer's solution did no longer make the ST-elevation in the direct unipolar electrocardiogram of a toad when sodium was increased twice as much in the solution. This result shows that the ST-elevation is not obtained if the ratio of sodium to potassium content is thus increased in the extracellular fluid. Prinzmetal *et al.*[25] have recently shown that the ST-deviation is related to the altered ratio of potassium to sodium in the extracellular space. These facts suggest that changes may occur in the membrane potential if the intra- or extracellular sodium is altered by the administration of insulin.

In our experiments the intracellular sodium was not directly determined. However, there were not such uniform changes in the sodium content of the insulin-glucose-Ringer's solution, as those seen in potassium. It is considered, therefore, that there is no secondary effect of sodium on the ST-elevation which

was produced by the potassium solution in our experiments.

2) The ratio of intra- to extracellular potassium and the effect of potassium solution on the ST-elevation in the heart treated with insulin.

The ratio of intra- to extracellular potassium is nearly 150 : 5 in the normal heart[21]. When it shows this value, the ST-elevation does not occur, as seen in the usual normal electrocardiogram. When the external potassium increases, so that the ratio changes to 150 : 7 for instance, the ST-elevation occurs, as observed in clinical and experimental hyperpotassemia. If the potassium is also increased in the inside of the cell and consequently the ratio approaches to normal value when the external potassium increases and the ST-elevation occurs, it is theoretically considered that the ST-elevation may be eliminated. Fig. 10.

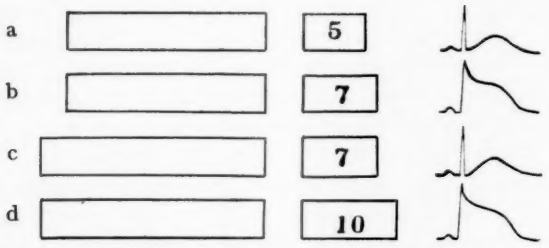

Fig. 10. The ratio of intra- to extracellular potassium and its relation to the ST-elevation (1);

(a) The normal ratio of 150 : 5. No change in the ST-segment, as seen in normal electrocardiogram.

(b) The extracellular potassium increases so that the ratio is 150 : 7 for instance, the ST-segment is elevated.

(c) To eliminate the ST-elevation in the situation b, it is necessary for the intracellular potassium to increase.

(d) If the external potassium increases more in the situation c and the ratio approaches to the same as that of b, the ST-elevation occurs.

indicates the relation. In other words, it is considered that in order to eliminate the ST-elevation it is necessary for the intracellular potassium to increase when the ST-elevation occurs subsequently to the increase of potassium in the outside of the cell. For this purpose, we tried to continue pouring the insulin-glucose-Ringer's solution on the heart surface, expecting potassium to transfer from the solution to the intracellular space, and then studied the electrocardiographic effect of potassium solutions on the heart.

The disappearance of the ST-elevation was not obtained by 6–7 mEq/L potassium solution immediately and soon after the insulin-glucose-Ringer's solution was applied to the epicardial surface. As time passed, however, 6–7 mEq/L potassium solution did not produce the ST-elevation. That is, after the

administration of insulin the disappearance of the ST-elevation was obtained by such potassium solution of the concentration which has always an effect on the normal intact heart surface. Thereafter a higher concentration was needed to produce the ST-elevation; the potassium solution of a certain concentration had no effect, while a higher concentration had the effect. The threshold value to produce the ST-elevation became higher gradually. These findings are explained by the observation that the intracellular potassium was gradually increasing after administration of insulin in our preliminary experiment. After the cessation of the administration, the threshold value was gradually lowered to normal. This suggests that the intracellular potassium was decreasing gradually and returned to the previous normal level.

Thus in the case of the increasing myocardial potassium, it is concluded that the ratio of intra- to extracellular potassium plays an important role in producing the ST-deviation.

The time of course of the elevated threshold was different in each case. This may be due to the different response of the individual heart muscle to insulin. The highest threshold level was also different in each case. But it seems higher in cases of higher potassium in the insulin-glucose-Ringer's solution than in the cases of the lower, This finding may be related to the fact that the heart in the former took up more potassium than in the latter.

3) The electrocardiographic effect of normal Ringer's solution on the heart when the intracellular potassium is increased.

As time passed after the administration of the insulin-glucose-Ringer's solution, the threshold value to produce the ST-elevation was higher. In other words, when the intracellular potassium is increased, the concentration of the potassium solution to produce the ST-elevation was higher. At this moment, the normal Ringer's solution, of course, produced no ST-elevation but the ST-depression was noted immediately after the Ringer's solution was poured.

After the cessation of the insulin-glucose-Ringer's solution, the threshold value was lowered gradually, and when it returned to normal the appearance of the ST-depression by the normal Ringer's solution was not obtained. This interesting electrocardiographic finding may be related to the altered ratio of intra- to extracellular potassium when the external potassium is normal and the intracellular potassium is increased. This concept is well supported by the observation that the ST-deppression appears immediately after pouring the normal Ringer's solution and its disappearance was found with the return of the threshold to normal.

The ratio is nearly 150 : 5 in the normal heart. When the intracellular potassium is increased, the external potassium also increased, and consequently the ratio approaches the normal value, the ST-elevation does not occur as

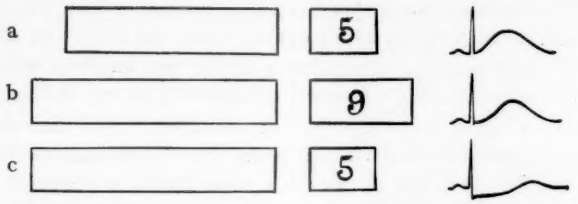

Fig. 11. The ratio of intra- to extracellular potassium and its relation to the ST-deviation (11);

(a) No change in the ST-segment, if the ratio is 150 : 5.

(b) The intracellular potassium increase with the increase of the external potassium. The ST-segment is not elevated. The ratio is the as that of a. Refer to the situation c in Fig. 10c.

(c) The ST-segment is depressed if the external potassium decreases to normal in the situation b.

observed in the present study. At that time, if the external potassium does not increase and remains normal, it is reasonably thought that the electrocardiographic finding ST-depression may be taken for an electrocardiogram of the increased myocardial potassium (Fig. 11). In this regard, Prinzmetal *et al.*[25] have recently shown that the ST-depression appeared within 1–2 seconds after injecting 5% glucose solution into the coronary artery immediately after ligation of the vessel, and they explained that this ST-depression was caused by increasing myocardial uptake of glucose and consequently of potassium. Thier observation and its explanation may be related to our finding.

SUMMARY and CONCLUSION

1) To examine the effect of insulin on the myocardial electrolytes the isolated heart of a toad was immersed in the insulin-glucose-Ringer's solution, and potassium and sodium contents of the solution were determined with time by use of a flamephotometory. Potassium content decreased with time. As to sodium, however, no uniform change was observed. This finding shows that the heart took up potassium from the surrounding solution in the presence of insulin and glucose.

2) Another study was performed on the electrocardiographic effect of potassium solution on a heart treated with insulin and glucose. The ventricular surface of a toad was applied continuously with the insulin-glucose-Ringer's solution, the heart was covered with potassium solutions of various concentrations, and the direct unipolar electrocardiogram was taken.

3) Immediately and soon after the administration of the insulin-glucose-Ringer's solution, the ST-elevation was obtained by 6–7 mEq/L potassium solution, which always produces it in the normal intact heart. As time passed, the

ST-elevation was not produced by 6–7 mEq/L potassium solution. It was necessary to use samples of higher concentration in order to produce the ST-elevation as the course progressed. After the cessation of the insulin-glucose-Ringer's solution, the threshold value of potassium concentration to produce the ST-elevation returned gradually to the previous normal level.

4) These findings may be explained by the supposition that the intracellular potassium is gradually increased after the administration of the insulin-Ringer's solution and is decreasing after the cessation. In the case of the increased myocardial potassium, the ratio of intra- to extracellular potassium plays an important role in the ST-deviation.

5) When the threshold value rose, the ST-depression was noted immediately if the normal Ringer's solution was used. This electrocardiographic finding may be taken for an electrocardiogram of the increased myocardial potassium when the external is normal.

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On the Applicability of the Oxygen Electrode to the Beating Dog Heart

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INTRODUCTION

In our investigations, tiny oxygen electrodes of open tip type were applied to the beating dog hearts.

Hitherto, J.J. Sayen *et al.* (1958)[1] have published their reports on these applications to the beating dog heart *in vivo*, though no continuous recording has appeared in their reports.

METHOD

An oxygen electrode circuit was demonstrated schematically in Fig. 1[2]. An inert electrode at the application of one volt or less, referring to an unpolarized electrode as the anode, could be polarized in the aqueous solution by the hydrogen ions and depolarized by the dissolved oxygen, becoming of an oxygen electrode.

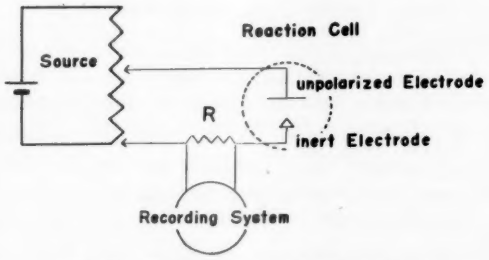

Fig. 1. Circuit diagram of polarometry.

For the oxygen electrode a platinum wire of 50 μ or less of the diameter was insulated into a glass capillary and used as an open tip type electrode. As shown in Fig. 2, it weighted about 100 mg. or less.

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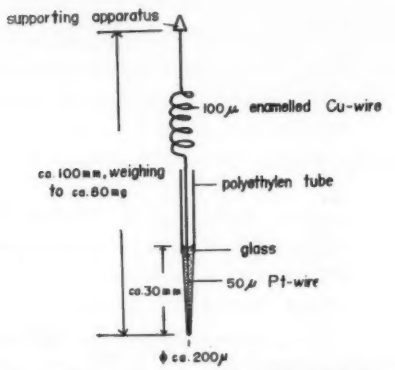

Fig. 2. The open-tip type of oxygen electrode used in this experiment.

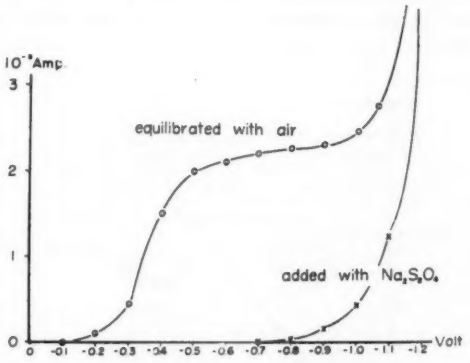

Fig. 3. The polarograms of both air-saturated and deoxygenated salines. Ordinate: Depolarizing currents. Abscissa: Applying voltages.

The depolarizing currents of about 10^{-8} ampère in the oxygen electrode circuits were amplified with an alternate current amplifier, intervened by a relay switch of 1 c/s,[3] and were recorded by Yokogawa's electro-magnetic oscillograph and/or Nihonkoden's cathode-ray-oscillograph.

The polarograms of the oxygen electrode circuits *in vitro* were shown in Fig. 3, for examples of an air-saturated saline and a deoxygenated saline. The polarograms were constructed by correlating the depolarizing currents in the term of galvanometric deflections with the applying voltages in the oxygen electrode circuits.

No change of the galvanometric deflections was seen at 400 to 800 milli-

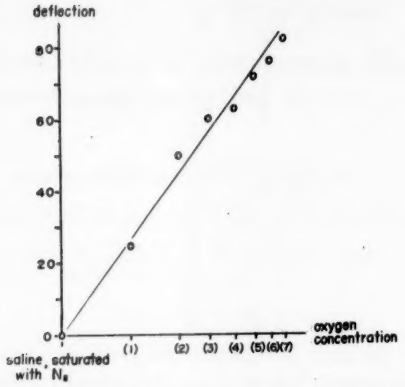

Fig. 4. A linear relationship between the galvanometric deflections and the oxygen concentrations in the oxygen electrode cells, where the oxygenated and the nitrogen-saturated salines were mixed in various proportions.

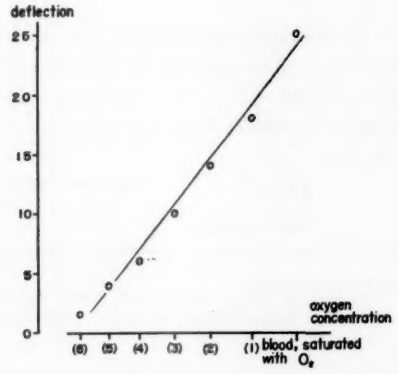

Fig. 5. A linear relationship between the galvanometric deflections and the oxygen concentrations in the oxygen electrode cells, where the oxygenated and the nitrogen-saturated of the heparinized whole bloods of the dogs were mixed in various proportions.

volts of the applied voltages, where the depolarization of the oxygen electrode as the cathode would be determined by the diffusion of the dissolved oxygen to the electrode. Since the diffusion velocity of the dissolved oxygen to the oxygen electrode should be determined by the concentration gradient between the solution and its nearest region to the cathode, the galvanometric deflections at the plateaux of the polarograms should be regarded as the concentrations of the dis-

solved oxygen in the solution.

That was indicated in Figs. 4 & 5. These figures were obtained by reading the galvanometric deflections at the plateaux ranges of the applied voltages, when the oxygenated and the nitrogen-saturated salines as well as the whole blood of a dog were mixed in the various proportions.

Thus we attempted the application of the oxygen electrode to the beating dog heart.

We anesthetized dogs (about 10 kg. of body weight) with intravenous and/or intraperiotneal administrations of nembutal (30 mg./kg. body weight), opened the chests according to the transsternal thoracotomy under artifitial respiration using bomb oxygen, and exposed the beating hearts hanging on the pericardium stitched to the edges of incised sternum.

An oxygen electrode was pricked into the mycoardium of the left ventricle. The movement of the tiny electrode was so completely coincident with the pulsation of the beating heart that its polarization seemed to be prevented from undesirable disturbances.

An Ag-AgCl electrode as the reference electrode was inserted into the chest wall.

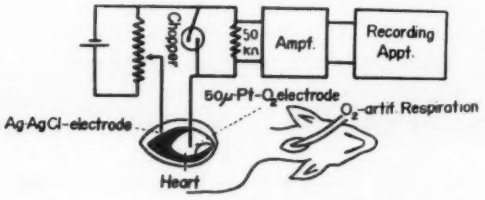

Fig. 6. The oxygen electrode cricuits *in vivo*.

The depolarizing currents of this circuit *in vivo*, as shown in Fig. 6, were amplified and recorded continuously together with changes of systemic blood pressures and respiratory changes of anesthetized dogs. Those changes were derived from mercury manometers which were intervened between basic oscillator circuits and recorders, according to T. Suzuki.[4]

RESULTS and DISCUSSION

The polarograms were taken of our oxygen electrodes in the beating dog hearts as seen in Fig. 7 for example. The plateaux could be reliable within errors of about 20%, even though the reasons why they were rather shortened and shifted to the lower ranges of the applied voltages than in the case of the buffered saline would remain to further investigations.

We examined responses of our oxygen electrodes in the beating cardiac

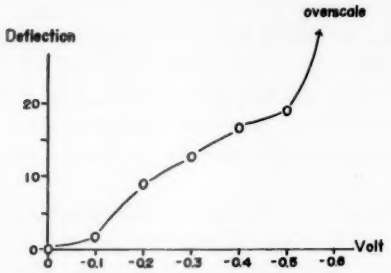

Fig. 7. A polarogram of an oxygen electrode in beating heart of a dog.

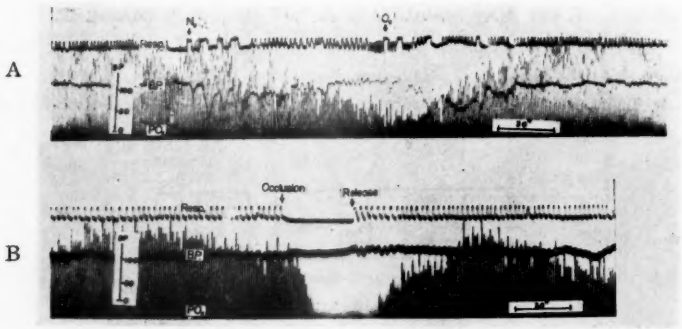

Fig. 8. Polarographic responses of the oxygen electrodes in beating dog hearts to anoxia, produced by (A) inhalation of nitrogen gas, (B) occlusion of respiratory tracts.

tissues to anoxia produced by occlusion of respiratoy tracts or inhalation of bomb nitrogen, supplying 400 to 500 mV on the circuits.

Examples of these results were demonstrated in Fig. 8. There were shown the respiratory changes uppermost, the downward deflections indicating expirations, and the systemic blood pressures as the mean values intermediately with a scale of mm. Hg. aside, and the polarographic responses were shown by the envelopes consisting of the vertically arranged lines and conveniently designated as Po_2.

When, in proceeding of anoxemic anoxia, the systemic blood pressure was not so greatly varied as failed to supply the blood into the coronary circulation, then the decreases of the depolarizing currents in the oxygen electrode of the beating heart might well reflect the lowering of oxygen tensions in the cardiac muscles as such. And also, when the anoxia was broken up, the oxygen electrode

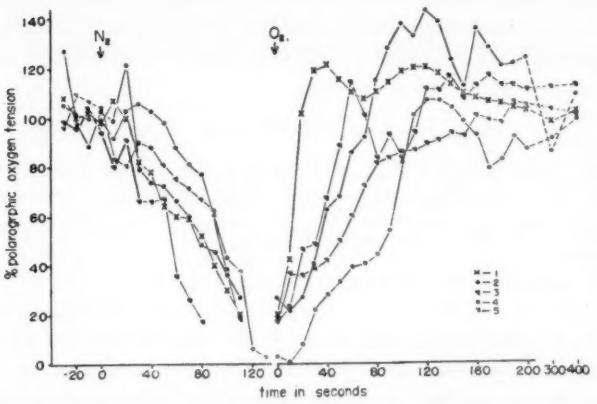

Fig. 9. The repeated polarographic responses of an oxygen electrode in a beating dog heart to anoxia, examining its applicability.
Each response was examined in the following order, 1, 2, 3, 4 & 5, at intervals of a few hours for about ten hours.

in the heart responded expectedly, increasing the depolarizing currents to the normal level.

Similar polarographic responses of the oxygen electrode had been observed by J.J. Sayen *et al.* (1958) in ligated regions of the dog hearts where the anoxia had been localized.

Meanwhile, because of those responses of our oxygen electrodes *in vivo*, it seemed that they could represent the changes *in situ* of the tissue oxygen tensions to which were applied a term of the polarographic oxygen tension according to J.J. Sayen *et al.* (1958).

As shown in Fig. 9, the polarographic responses of an oxygen electrode in a beating dog heart to anoxia produced by inhalation of nitrogen gas were repeatedly examined at intervals of a few hours for about ten hours or more. In the figure, the mean normal level of the polarographic oxygen tensions in the left chamber walls of the beating dog heart was shown as 100%, and the minimal value of the depolarizing currents during the nitrogen inhalation was regarded as 0%. Pracitcally, 0% of the polarographic oxygen tension might be corresponded to about 10% or less of the oxygen saturation, according to R.M. Berne (1957, 1958)[5] and H. G. Swann (1953).[6] Thus, Fig. 9 demonstrated that our oxygen electrodes were safely and accurately reacting to the oxygen contents even in pulsating cardiac tissues.

It seemed that our oxygen electrodes, which were made of a thin and fine platinum wire (50 μ of the diameter), were adequate to measure changes of the

oxygen contents in living cardiac tissues. Because most workers[7)] used a platinum wire of much larger diameter (200 to 500 μ) for their oxygen electrodes than in the case of ours, probably their polarizations on the supplying voltages might be more sluggish and incomplete.

SUMMARY

1) An inert electrode at the application of one volt or less, referring to an unpolarized electrode as the anode, could be polarized in the aqueous solution by the hydrogen ions and depolarized by the dissolved oxygen, becoming of an oxygen electrode.

2) We examined the applicability of the oxygen electrode to the beating dog heart.

3) The depolarizing currents of about 10^{-8} ampère in a tiny platinum electrode of open tip type at the oxygen electrode were amplified to make continuous recordings possible. It seemed that they could represent the polarographic oxygen tensions (Sayen *et al*) in living cardiac tissues, because of the presence of the plateau in the polarogram *in vivo* and the expected responsiveness of our oxygen electrodes in beating dog hearts to anoxia.

We wish to express our thanks to Prof. T. Suzuki, Department of Applied Physiology, Tohoku University, Sendai, Japan, in whose laboratory most of our works were carried out.

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Pantothenic Acid Content of Human Blood

By

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(Received for publication, November 9, 1960)

A microbiological method for the assay of pantothenic acid was devised by Pennington *et al.*[1] in 1940 and was later modified by Stanberg and Esmond[2] (1940) and by Pearson, *et al.*[3] (1944) for human blood determination. In 1944, Skeggs, *et al.*[4] showed that pantothenic acid in tissues existed in bound forms which were not available for the growth of the test organism, and in 1948, Kaplan and Lipmann showed that most of the pantothenic acid in blood occurred as a Coenzyme A in red cells and only a small part of it was free in the plasma. A useful study on the enzymatic liberation of bound pantothenic acid by liver amidase and intestinal phosphatase was reported by Neilands *et al.*[6]

In our previous report,[7] we pointed out that the enzymatic liberation of pantothenic acid in blood by acidic phosphatase enzymes such as takadiastase-papain was incomplete.

In the present study, we have used the technique of Neilands, *et al.* for the determination of pantothenic acid content in blood in order to obtain more complete enzymatic liberation of pantothenic acid.

EXPERIMENTAL

Blood specimens were obtained from 78 farm-villagers aged 30–69 living in north-east Japan. The age and sex constitution of the group is shown in Table I.

TABLE I. Age and Sex Constitution of Subjects

Age \ Sex	Male	Female	Both
30 - 39 yr.	9	9	18
40 - 49	9	14	23
50 - 59	10	8	18
60 - 69	9	10	19
total	37	41	78

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The pantothenic acid contents of 8 specimens (five from males and three from females) were measured by two methods: (1) takadiastase-papain method reported previously and (II) modified Neilands' method described as follows.

A measured quantity (1.0 ml) of blood was taken in each tube and haemolysed by adding about 5 ml of water and the pH was adjusted to 8.3 with trisaminomethane buffer solution. Intestinal phosphatase solution[6] (0.1 ml) and chicken liver enzyme solution[8] (0.2 ml) were added and the mixture was incubated at 37°C for 10–12 hours. Enzymatic liberation was then stopped by boiling on a water bath for 5 minutes and 2.5 ml of 10% trichloroacetic acid was added in order to separate residual protein. The pH was adjusted to 6.8, and the mixture was made up to 50 ml and then centrifuged at 3000 r.p.m. for 5 minutes. Microbiological determination was carried out on 0.5, 1.0 and 2.0 ml of supernatant aliquots.

RESULTS

Table II presents a comparison of pantothenic acid content in blood estimated by (I) the takadiastase-papain method, and (II) the modified Neilands' method.

TABLE II. Comparison of Pantothenic Acid Content in Blood between Two Methods (I and II)

Name	Age	Sex	I	II	II/I
S. K.	28	Male	67γ%	135γ%	2.01
K. I.	29	〃	87	108	1.40
S. K.[2]	31	〃	73	110	1.51
Y. K.	32	〃	81	119	1.47
E. A.	33	〃	59	115	1.95
K. S.	22	Female	61	92	1.51
S. T.	39	〃	41	47	1.15
H. T.	40	〃	67	116	1.73
Mean (95% confidence limits)			67.0 (55.2–78.8)	105.3 (83.2–127.4)	1.60 (1.31–1.89)

TABLE III. Pantothenic Acid Content in Human Blood by Age and Sex

Age \ Sex	Male	Female
30 - 39 yr.	117.4 γ% (87.2–147.6)	109.6 γ% (90.1–129.1)
40 - 49	96.9 (76.9–116.9)	100.6 (87.8–113.4)
50 - 59	94.0 (69.8–118.2)	90.8 (80.7–100.9)
60 - 69	94.2 (84.6–103.6)	87.1 (74.0–100.2)

Numerals in parentheses denote 95% confidence limits of means.

Mean values and 95% confidence limits were 67.0 (55.2–78.8) and 105.3 (83.2–127.4)γ/100 ml respectively and the ratio of (II) to (I) was 1.60 (1.31–1.89).

Table III presents mean pantothenic acid levels estimated by Neilands' method of the 78 farm-villagers divided according to age and sex. The values tend to fall with increasing age, especially among females, but numbers are too small to give significant differences.

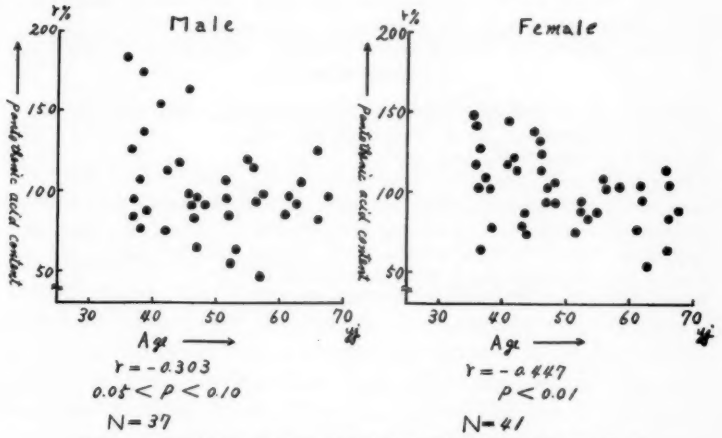

Fig. 1. Correlation between Pantothenic Acid Content and Age.

Fig. 1. shows the correlation between pantothenic acid content and age. The correlation coefficients are –0.303 ($P<0.10$) for males and –0.447($P<0.01$) for females.

DISCUSSION

Although the role of pantothenic acid in tissue metabolism has been appreciated in recent years, there has hitherto been no accurate information on the pantothenic acid level in blood. The present findings may not be typical of all human communities because our subjects are farm-villagers, most of whom are engaged in farming and whose food consists largely of rice.

The observation that aging is associated with a lowering of the blood pantothenic acid level is interesting in view of the recommendation to give pantothenic acid to old people to prevent decline of liver function. The fact that the association with age is more marked in females than in males suggests that there may be some relationship between the synthesis of steroi hormones and the pantothenic acid level in the body[9].

SUMMARY

The blood pantothenic acid levels of 78 farm-villagers aged 30–69 yr. (37 males and 41 females) were estimated and were found to fall with advancing age. This tendency was more marked for females than for males.

ACKNOWLEDGEMENT

We wish to thank Professor E. Takahashi and Assistant Professor K. Katô who have always given valuable suggestions and revisions.

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Tohoku J. Exper. Med., 1961, 74, 69–75

Fractional Estimation of Glucosamine and Galactosamine by Paper Chromatography. Third Report.

By

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(Received for publication, January 17,1961)

The author[1),2)] reported previously the methods of fractional estimation of glucosamine and galactosamine by paper chromatography. In those methods, Elson-Morgan reaction (a modification of Rondle and Morgan's procedure) was adapted to estimate the hexosamines.

Dische[3)] stated that Dische-Borenfreund's indole-HCl reaction[4)] for 2,5-anhydrohexoses formed after deamination of hexosamines was about five times as sensitive as the Elson-Morgan reaction and appeared to be less affected by impurities even in crude preparations of polysaccharides, and also that in highly purified blood group substances, the values obtained by the two methods were found to be in good agreement, whereas when applied to acid mucopolysaccharides such as hyaluronic acid and chondroitinsulfuric acids, great discrepancies were found in the values obtained by the two methods.

In this paper, the author applied the Dische-Borenfreund reaction to estimate differentially glucosamine and galactosamine in purified blood group mucopolysaccharide prepared from pig gastric mucus, and found that this method gave more reproducible results than the procedure previously used.

As can be seen in experimental part (Table I and Fig. 2), several hydrolysis experiments with 4 N and 6 N hydrochloric acid indicated that the ratio of free glucosamine to free galactosamine released from the sample was approximately equal, although in two cases of these experiments some of the hexosamines were not liberated in free form.

ANALYTICAL TECHNIQUES

Analysis of Total Hexosamines

Hydrolysis is performed according to the method[2)] previously reported and hexosamines are estimated principally by the Dische-Borenfreund's procedure.[4)]

(1) An amount of sample containing 30～200 μg. of hexosamines in total is weighed in a stoppered glass tube graduated at 5 ml., and hydrolyzed with

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1 ml. of 4 N hydrochloric acid in a boiling water bath for 4 hr..[5] The hydrolysate is cooled in ice-water. The solution was added with a drop of 0.5% phenolphthalein solution in 50% alcohol, then neutralized with 0.75 ml. of 4 N sodium hydroxide and a required quantity of 0.5 N sodium hydroxide to distinct red color, followed by immediate addition of 0.3 N hydrochloric acid until the red color is just disappeared. Final volume is made up to 5 ml. with water, and filtered, if necessary.

(2) To 0.5 ml. of the above solution are added 0.5 ml. of 5% aqueous sodium nitrite and 0.5 ml. of 33% solution of acetic acid. The test tube is shaken and stood for 30 min. under occasional shaking. After that, 0.5 ml. of 12.5% aqueous ammonium sulfamate was added to remove excess nitrous acid, followed by repeated shaking for further 30 min..

(3) To the solution are added 2 ml. of 5% hydrochloric acid and 0.2 ml. of 1% solution of indole in alcohol. The tube is then immersed for 5 min. in a vigorously boiling water bath, followed by cooling in water at room temperature. The solution is then added with 2 ml. of alcohol and shaken.

The difference of the optical density at 492 mμ and 550 mμ (D_{492}–D_{550}) is read against the blank. As the reference, a standard hexosamine and a control sample without deamination, which are treated in the same way, are required.

(4) Provisional total hexosamine content in the sample can be given either as glucosamine or as galactosamine: under the mentioned conditions of analysis the reading of the D_{492}–D_{550} for galactosamine was 96% in average to that of the same amount of glucosamine.

Fractional Determination of Glucosamine and Galactosamine

(A) Provisional total amount of hexosamines is estimated as above.

(B) A suitable amount of sample is hydrolyzed with 2 ml. of 6 N hydrochloric acid for 15~24 hr., and the acid is removed as much as possible by repeated condensation to dryness of the solution in vacuo over anhydrous calcium chloride and solid caustic sodium hydroxide, with occasional addition of water. The residue is dissolved in water and the solution is filtered prior to final condensation. The final residue is dissolved in 0.05~0.1 ml. of water. The solution is sampled at positions marked × on a Toyo Roshi filter paper No. 3 (12 × 56 cm. square) shown in Fig. 1. The sample is chromatographed and treated same way as reported[2] previously: The sample is irrigated in descending way with the solvent mixture of n-butanol-pyridine-dilute hydrochloric acid (5 : 3 : 2 by volume) (pH 5.6~6.0) at 26~28°C. After irrigation for a suitable period of time (approx. 48 hr.), the paper is dried in air and washed through dry ether. Sections 1~3 are cut asunder, and one of them is sprayed with the aniline hydrogen phthalate

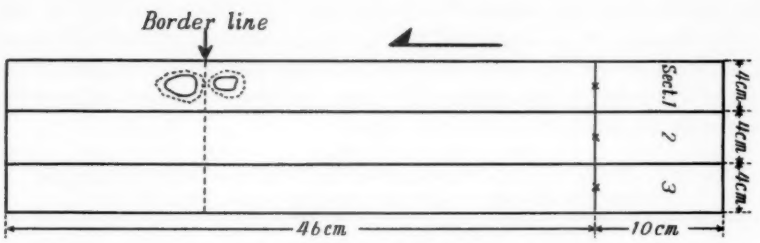

Fig. 1. Paper sliver used in the experiments.

reagent of partridge to distinguish two spots of the hexosamines. A straight horizontal line corresponding to the border line of the two sports on section 1 is pencilled on sections 2 and 3. Then, 15~20 consecutive parallel lines are pencilled at 0.5 cm intervals on both sides of the provisional border line on each of these sections, followed by scissoring along these lines. The small-slivers thus obtained are separately cut into pieces, put into test tubes. To each tube, 0.5 ml. of water is added and hexosamine content is assayed directly in the tube by the above method. The amount of the hexosamine in each tube is plotted on a section-paper to recognize the true border between glucosamine and galactosamine. By this way, the ratio of free glucosamine to free galactosamine can be obtained from the graph.

(C) Calculation. The contents of glucosamine (x), galactosamine (y) and the true total hexosamines (z) in the sample analyzed are calculated in terms of percentage by the following equations:

$$x\left(1+\frac{0.96}{r}\right)=T, \quad y=\frac{x}{r} \quad \text{and} \quad z=x+y,$$

where T is the per cent of the provisional total hexosamines expressed as glucosamine in the sample, r is the ratio of glucosamine to galactosamine in the sample examined and 0.96 is an empirical coefficient for galactosamine against glucosamine.

COMMENT

Purified blood group AO mucopolysaccharide prepared from pig gastric mucus [N 3.6%, total hexosamine 33.7% (by Dische-Borenfreund reaction) and 30.5% (by Elson-Morgan reaction), glucosamine/galactosamine 2.1, galactose 29.0%, L-fucose 14.0%, sialic acid 2.4%, ash 3.0%, Groups A and O active at dilutions of $1:1\times10^7$ and $1:5\times10^4$] was hydrolyzed in a boiling water bath by either of following several ways: (1) 4N HCl for 4 hr., (2) 6N HCl for 4 hr., (3) 6N HCl for 8 hr., (4) 6N HCl for 15 hr., (5) 6N HCl for 24 hr.. Then, these hydrolysates were analyzed by the procedure described above in (B). In these experiments, examinations were carried out with paper slivers covering whole

area starting from the sampled line to the end of the glucosamine spot in order to know the ratio of the amount of free hexosamines to that of total hexosamines on the paper slivers, for Dische-Borenfreund reaction is positive for either reducing and non-reducing hexosamines provided that they have free amino group. The results are shown in Table I and Fig. 2.

TABLE I. Ratios of Free Glucosamine to Free Galactosamine and Percentages of Free Hexosamines to Total Hexosamines on the Paper Slivers.

No. of experiments	Conditions of hydrolysis in a boiling water bath		Free glucosamine / Free galactosamine (ratio)	Free hexosamines / Total hexosamines (%)
	Normality of HCl used	Period of time (hr.)		
1	4	4	2.1	75.8
2	6	4	2.1	89.0
3	6	8	2.0	99.4
4	6	15	2.1	99.2
5	6	24	2.1	99.1

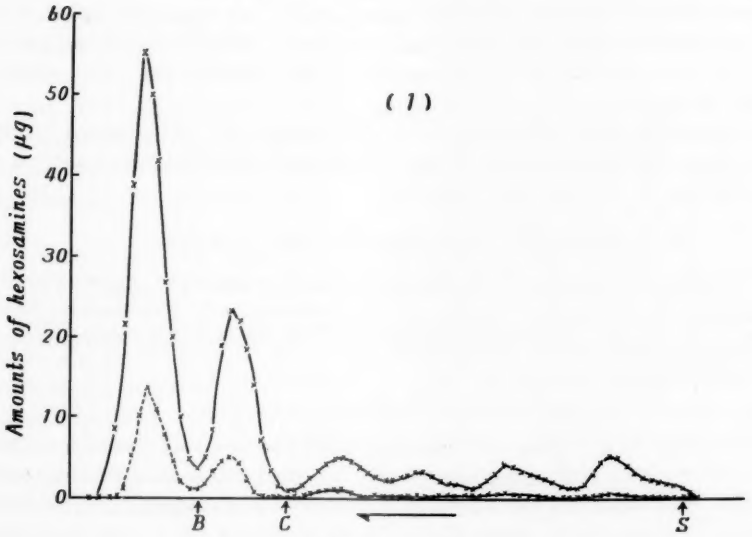

Fig. 2 (1)

Fig. 2. Distribution of hexosamines on the paper slivers of the experiments shown in Table I.
S: sampled line, B: true border line between free glucosamine and free galactosamine, C: border line between free galactosamine and combined hexosamines.
×—×—×—: estimated by Dische-Borenfreund reaction.
×---×---×---: estimated by Elson-Morgan reaction.

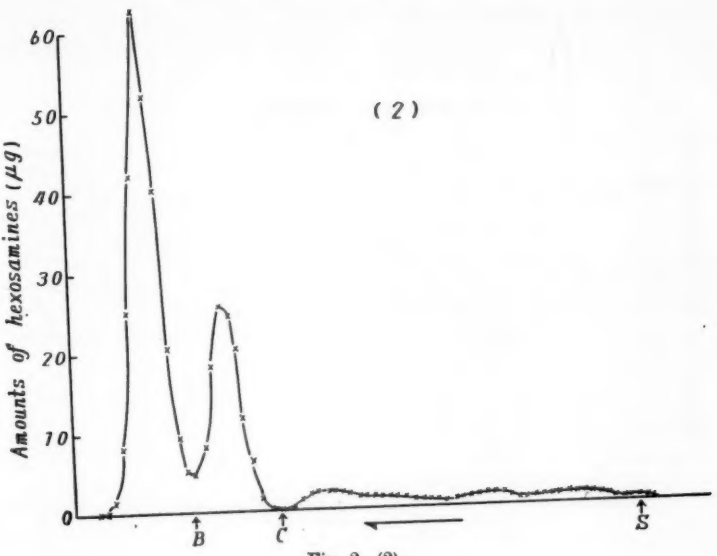
(2)
Amounts of hexosamines (μg)
60
50
40
30
20
10
0
B
C
S

Fig. 2 (2)

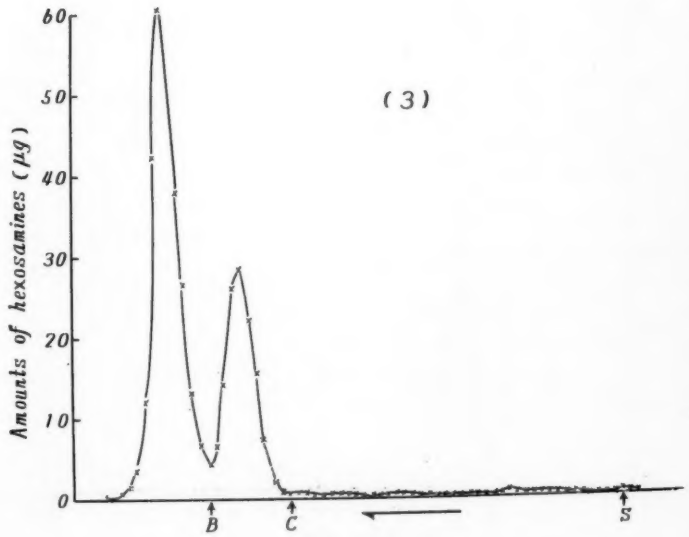
(3)
Amounts of hexosamines (μg)
60
50
40
30
20
10
0
B
C
S

Fig. 2 (3)

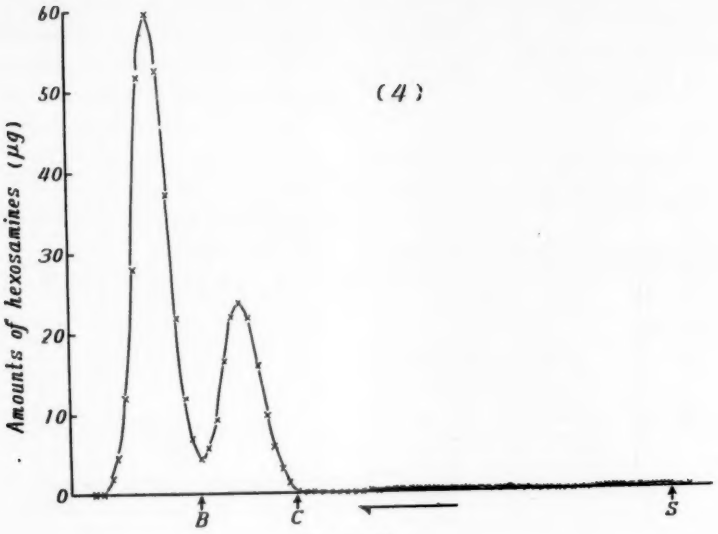
(4)
Amounts of hexosamines (μg)
60
50
40
30
20
10
0
B
C
S

Fig. 2 (4)

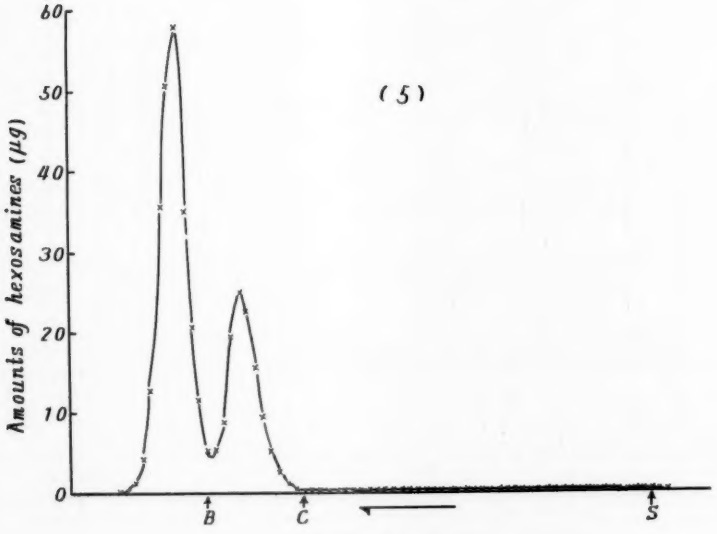
(5)
Amounts of hexosamines (μg)
60
50
40
30
20
10
0
B
C
S

Fig. 2 (5)

This work was supported by a grant for scientific researches from the Ministry of Education.

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5) As already reported,[2] when estimation of the total hexosamines was carried out by Elson-Morgan reaction, purified blood group mucopolysaccharide, hyaluronic acid (mucoitin) and sodium chondroitin sulfate gave the maximal values by the hydrolysis with 4 N hydrochloric acid for 4 hr. in a boiling water bath.

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Hue Shift with Intensity (Bezo'd-Brücke Phenomenon) as Studied by the "Zeta-Method"

By

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The hue of monochromatic light usually changes when its intensity is varied. Such a hue shift with the stimulus intensity is known as the "Bezold-Brücke phenomenon" (B.B.P.).[1)]

The colors corresponding to longer wave-lengths (red, orange and yellow-green) tend towards yellow, and those corresponding to shorter wave-lengths (blue-green and violet) change towards blue, as the stimulus intensity is raised. But some monochromatic lights in the yellow, green, and blue parts of the spectrum are invariable in hue. von Bezold stated that all the hues in the spectrum were changed towards one of three hues (red, green, and violet) as retinal illuminance was made lower and lower, though this statement is not entirely correct. Purdy[3)] studied quantitatively this effect with the method of hue matching and Judd[4,5)] tried to explain and calculate quantitatively this phenomenon in terms of the Hering's opponent color theory. In the present investigation, this phenomenon was studied with the so-called "Zeta-method" of Motokawa in which electrical phosphenes are taken as an index of retinal excitation.

METHOD

It is well-known that a sensation of light can be evoked by electrical stimulation of the eye. The electrical excitability of the eye can be measured with this sensation as an index. After exposure to light, the electrical excitability (the reciprocal of the threshold) of the dark-adapted eye rises to a maximum following an initial depression, and then decreases gradually to its resting level. The magnitude of supernormality is a function of the intensity[6)]. In order to express the supernormality quantitatively, a quantity "ζ" was introduced by the following formula:

$\zeta = 100(E - E_0)/E_0$, where E and E_0 denote the electrical excitabilities determined with and without preillumination.

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The procedure and arrangement used for determination of electrical thresholds were essentially the same as described in the previous papers[7,8].

After a preliminary dark adaptation of about 20 minutes the eye was exposed to a light stimulus, white or colored, lasting 2 sec., and at various moments such as 0.5, 0.75, 1.0 sec., etc., after removal of the light, an electrical test stimulus, a rectangular pulse of 100 msec. in duration, was applied to the eye. Tests were repeated every 15 seconds. An applied voltage of a few volts usually caused a distinct phosphene. Then, the stimulus voltage was reduced step by step until the subject found it difficult to discriminate an electrical phosphene from the background of intrinsic light and he demanded a comparison procedure. This procedure consisted of delivering two stimuli at an interval of several seconds in random order, one in question and the other a control one of zero voltage. The subject was requested to answer the question which the stronger one was, and informed every time whether his answer was right or wrong. The same procedure was repeated with voltages reduced by about one per cent step by step, until the subject could no more discriminate both stimuli. If the answer was wrong, tests with the same voltage were repeated three times. When the subject succeeded twice out of three trials, the voltage was reduced further, and the latest right value was adopted as a threshold (see notation "a" in Table I).

The subject was allowed to request a further trials when he thought that his failure had been due to some accidental disturbance of attention or due to wrong judgment in spite of a fairly distinct difference perceived between the effects of two stimuli (see notation "b" in Table I). When the subject failed to give right answers successively in the first three trials after commencement of the comparison procedure, no further test was made, because the comparison procedure should have been begun earlier. Thus, the measurement had to be renewed (see notation "c" in Table I). The stimulus voltage was controlled by a rheostat.

Examples of protocols for gradation of the stimulating voltage are presented in Table I.

The electrodes used were a pair of silver plates of 2×1.5 cm^2 in size, one placed on the middle of the forehead as the cathode and the other on the temple as the anode.

Monochromatic lights of various wave-lengths were produced with a series of interference filters and were made equal with respect to energy. The percentage transmission of a red filter (640 mμ) was 4.5 per cent. For preillumination, a patch of opaque glass, 2° in visual angle, was used upon which the colored lights were projected. The experiment was done at the central view. The white light used as source was an incandescent lamp with tungsten filaments of about 2850°K in color temperature which was measured with an optical pyrometer. The intensity of illumination in front of the opaque glass was 75,000 lux. For reduction of intensity, neutral tint filters of various transmission

TABLE I. Protocols of Threshold Determination. Vr Denotes Stimulus Voltage in Volts at Resting Level. $V_{1.0}$, $V_{1.25}$, etc. Denote Those Applied 1.0, 1.25 sec., etc., after End of Preillumination with Light of 560 mμ Respectively.

V_r	Response	$V_{1.0}$	Response	$V_{1.25}$	Response	$V_{1.25}$	Response	$V_{1.5}$	Response	$V_{1.75}$	Response	$V_{2.0}$	Response
0.750	Distinct	0.522	Distinct	0.500	Distinct	0.500	Weak	0.462	Weak	0.500	Weak	0.522	Weak
0.649	Weak	0.480	Weak	0.449	Faint	0.453	Faint	0.421	Weak	0.453	Weak	0.480	Faint
0.600	Faint	0.471	Faint	0.440	Very faint	0.444	Very faint	0.410	Faint	0.440	Faint	0.471	Faint
0.572	Very faint	Comparison		Comparison		Comparison		Comparison		Comparison		Comparison	
Comparison		0.471	+	0.436[c]	+	0.440	+	0.407	+	0.436	+	0.462	+
0.565[b]	−	0.466	+	0.432	+	0.436	+	0.403	+	0.432	+	0.457	+
0.558	−	0.462	+	0.428	−, −	0.432[a]	−, +, +	0.399	−	0.429	+	0.453	+
0.552	+	**0.457**	**+**	0.425	−	**0.429**	**+**	**0.395**	**+, +**	**0.425**	**+**	**0.449**	**+**
0.545	+	0.453	0	0.421	−	0.425	−	0.391	−, −	0.421	0	0.444	−, 0
0.539	**+**	0.449	0	Further trials		0.421	0	0.387	0	0.417	0	0.440	0
0.533	0, +, −	0.444	0	were given up		0.417	0	0.383	0	Step down 5%		Step down 5%	
0.527	0	Step down 5%		to begin from		Step down 5%		Step down 5%		0.400	0	0.421	0
0.522	0	0.425	0	first step.		0.400	0	0.364	0				
Step down 5%													
0.500	0												

Plus : Right Answer; Minus : Wrong Answer; Zero : No Answer or Equal Phosphenes to Both Stimuli.
See Text as to Notations a, b, and c.

factors were used.

RESULTS

1. Reproducibility of the ζ-time curve

The ζ-time curves obtained from one and the same subject under the same experimental conditions, coincided very satisfactorily, although they were obtained in different sessions. Examples are shown in Table II. In this case, the ζ values could be reproduced within experimental errors of 1.5 in terms of ζ, although the experiments extended over 4 months. The accuracy in this case is of the same order as in the previous experiments by Kohata *et al.*[10] The electrical threshold itself showed daily remarkable variations, but ζ values remained almost constant.

TABLE II. Reproducibility of ζ Values. $\zeta_{1.0}$, $\zeta_{1.25}$, etc. Denote ζ Values at 1.0, 1.25 sec., etc., after Termination of Preillumination with Light of 560 mμ Respectively. V is Threshold Voltage in Volts at Resting Level.

Relative intensity of light	V	$\zeta_{1.00}$	$\zeta_{1.25}$	$\zeta_{1.50}$	$\zeta_{1.75}$	$\zeta_{2.00}$	Date
100	0.572	36.9	45.1	53.9	46.1	36.4	July 13
	0.505	37.9	46.8	54.1	44.6	39.2	July 14
	0.552	37.9	45.8	55.8	43.1	38.8	Sep. 30
	0.552	37.6	47.9	56.3	45.8	40.2	Nov. 4
	0.539	36.8	45.7	55.7	44.3	38.9	Nov. 5
1	0.545	19.3	25.2	33.8	26.2	19.3	July 13
	0.524	19.8	24.2	36.3	26.8	18.7	July 14
	0.552	17.3	26.3	37.9	27.6	18.4	Sep. 30
	0.552	18.3	26.3	38.8	26.3	19.6	Nov. 4
	0.539	17.9	25.6	36.5	26.8	20.0	Nov. 5

2. ζ-Time curves for various monochromatic lights

By plotting ζ values against the time elapsed from the end of preillumination, we obtained ζ-time curves representing the time-courses of supernormality. In the following experiments, a pair of ζ-time curves, one for a relative intensity 1 and the other for a relative intensity 100 were obtained for each wave-length. The results are represented in Figs. 1 and 2. The time from the end of preillumination to the maximum of the ζ-time curve will be called a "crest time". In general, the crest time is shorter for longer wave-lengths and longer for shorter one, but it is not always the same for one and the same wave-length when there is a difference in intensity.

As seen in Figs. 1 and 2, the crest times of the two ζ-time curves obtained at different intensity levels are almost identical in three regions of the spectrum,

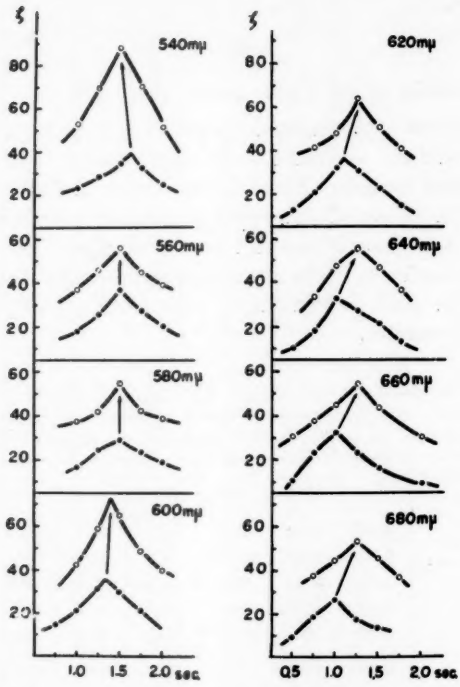

Fig. 1. ζ-time curves for monochromatic lights of various wave-lengths. Lower curves marked by dots were obtained at relative intensity 1 and higher ones at intensity 100 (without neutral tint filter).

namely, 400–420, 480–500, and 560–600 mμ. In these regions the line combining the two crests of the ζ-time curves is almost vertical. In the other regions, the crest time is different according to the intensity so that the line combining the peaks of the two ζ-time curves is inclined in either direction.

In order to express this relation more clearly, the diagram in Fig. 3 was constructed, in which the crest times are plotted as ordinates against wave-lengths. The crest times for the relative intensity 1 are represented by dots, and those for the relative intensity 100 by open circles. As seen from this diagram, the change of the crest time with wave-length is not steady throughout the whole range of wave-lengths, but is steeper in two regions, 480–500, and 560–600 mμ than in any other regions. This relation has been confirmed repeatedly in the previous studies by Motokawa *et al.*[11,12] It was pointed out by Motokawa that the wave-length discrimination is better in these regions in which the crest time

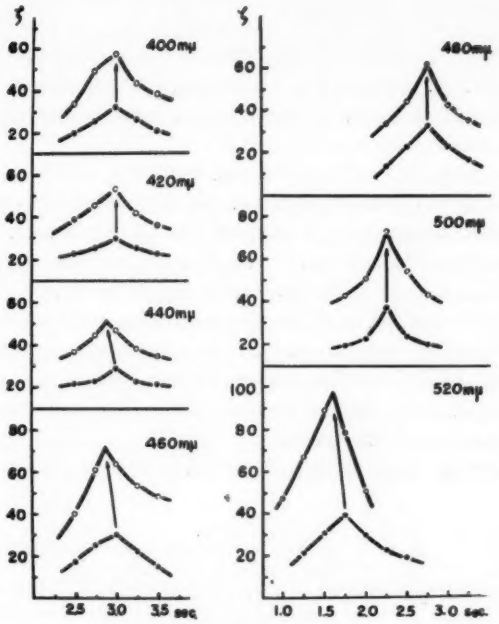

Fig. 2. Same as in Fig. 1.

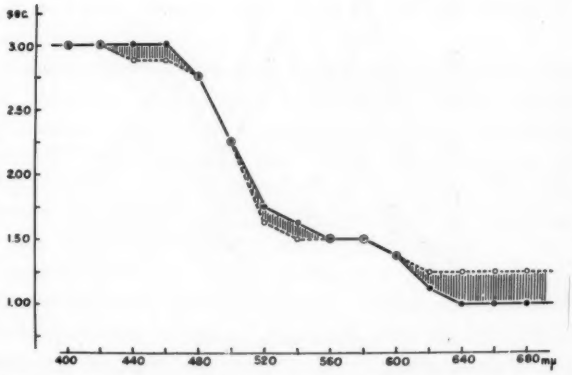

Fig. 3. Diagram representing relation between crest times and wave-lengths. Continuous curve marked by dots refers to intensity 1 and broken one marked with open circles to intensity 100. Regions where hue shifts occur are hatched.

changes abruptly with wave-length.

3. Change in crest time with intensity.

The two kinds of curves in Fig. 3 coincided in three regions of wave-lengths, that is, 400–420, 480–500, and 560–600 mμ, and these regions seem to correspond with those of invariant color in the B.B.P.

In the two parts from 420 to 480 and from 500 to 560 mμ, the crest time becomes shorter as the intensity is raised, but a change in the reverse direction occurs over the wave-lengths longer than 600 mμ. At the red end of the spectrum, for instance, the crest time shifted from 1.0 sec. to 1.25 sec., when the intensity was raised from 1 to 100. The crest time of 1.25 would be obtained for the orange light of 610 mμ and unit intensity, as will be predicted from the diagram in Fig. 3. This means that the red light of intensity 100 can be matched with the orange light of unit intensity, taking the crest time as an index.

Such a procedure is quite equivalent to the hue matching carried out in Purdy's experiment to study the B.B.P.

As stated above, the crest time shortened by raising the intensity form 1 to

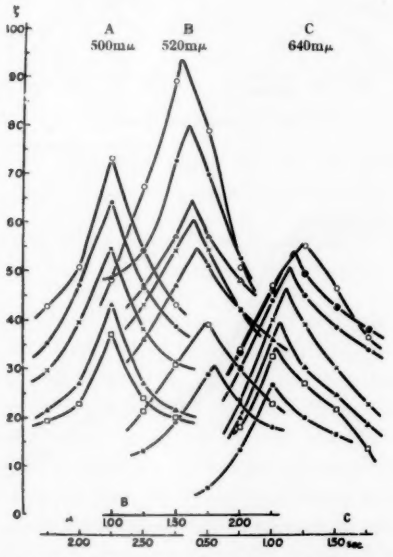

Fig. 4. Steady shifts of crest caused by variations of intensity in small steps. No shift in group A, shift to left in B, and shift to right in C. Circles, double circles, dots, triangles, crosses, filled triangles, squares, and filled squares refer to relative intensity 100, 49, 43, 17, 11, 4, 1, and 0.5 respectively.

100 in the other two regions, 420–480, and 500–560 mμ. It is obvious from the diagram that a monochromatic light of a higher intensity in those regions can be matched with another monochromatic light of lower intensity and longer wave-length. The same relation was observed by Purdy in his color matching experiments.

In the experiments mentioned above only two greatly different levels of intensity were used. The question now arises as to whether or not a continuous change occurs in crest time as the intensity is changed continuously.

With three selected wave-lengths, i.e., 500, 520, and 640 mμ, the intensity of monochromatic light was varied in small steps, and three sets of ζ-time curves in Fig. 4 were obtained. In the set A for 500 mμ, no horizontal displacement of the crest took place as the intensity was raised, and this experiment has confirmed again that the light of 500 mμ is invariant in hue. In the set B for 520 mμ, the crest time became shorter and shorter with increasing intensity, while it was lengthened continuously in the set C for 640 mμ. From these experiments it is likely that the change of the crest time is continuous as the stimulus intensity is continuously changed.

4. The crest time as a counterpart of the hue.

When retinal illuminance is increased, a decrease in saturation and an increase in luminosity occur besides such a hue shift as stated in the B.B.P. From the experiments stated above it seems that the effect of the intensity on the crest time is comparable to the hue shift in the B.B.P., but it is also possible that the change in crest time depends not so much on the hue shift as on changes in saturation and luminosity.

In the following experiments, a monochromatic light which would show a color shift on increasing its intensity was mixed with various amounts of white light so that a series of light with varying saturation and luminosity, but fixed hue, was obtained. The white light used here in this experiment was from the source C of I.C.I. and its intensity ranged from 37 to 912 lux. The monochromatic lights of 500, 520, and 640 mμ were 17 in relative intensity. Both lights, colored and white, were mixed by means of a half-mirror.

If the crest time was to depend on the hue, but not on saturation and luminosity, it was expected that the crest time would remain unaltered and independent of the amounts of white light mixed with the monochromatic light. As a matter of fact, the crest time was found entirely independent of the amounts of white light added to, as illustrated by the left set of ζ-time curves in Fig. 5, in which the bottom curve represents a control or the curve for the pure monochromatic light of 520 mμ. The same held for monochromatic lights of invariant color, as shown in the right set of curves in the same figure.

The fourth curve from bottom in the right set showed two elevations, one at

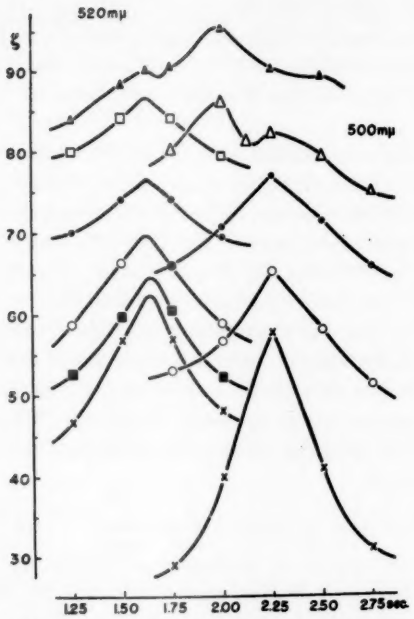

Fig. 5. ξ-time curves obtained with mixtures of monochromatic light of 520 or 500 mμ and varying amounts of white light. Two curves at bottom marked by crosses represent controls for pure monochromatic lights. Filled triangles, squares, triangles, dots, open circles, and filled squares refer to amounts of white light added : 912, 638, 447, 313, 76, and 37 lux respectively.

2.25 seconds, and the other 2.00 seconds. The former is obviously the crest characteristic for the monochromatic light of 500 mμ, and the elevation at 2.00 seconds is characteristic of the white light. When the amounts of white light added to the monochromatic light are small, the peak at 2.25 seconds is predominant so that no peak corresponding to the white component of the mixture appears. If the amounts of white light mixed are greater than a certain limit, its proper peak appears besides the one characteristic of the monochromatic component. When desaturation caused by the addition of the white light is so strong that no coloration is perceived, there is, of course, no peak for the monochromatic component.

One may suppose that the colorimetric purity may be determined by measuring the threshold amount of white light necessary to add to make the peak for the monochromatic component just disappear, but such a method is not so practical, because the measurement of such a threshold is tedious and difficult.

There is another very practical method to determine the colorimetric purity, as was shown by Onoki[13]. In this method the property of retinal induction is utilized which is very sensitive to the colorimetric purity.

DISCUSSION

We fear that in our experiments the ζ-time curves were drawn with too small a number of points to determine their crest times accurately, but each point could be reproduced satisfactorily, as has been shown above. It was sometimes difficult to determine the peak of the ζ-time curve, because points around the peak were too dispersed. In such a case the peak had to be drawn manually so that it might fit in with the general trend of the curve. In order to avoid any biases of the experimenter, the manual drawing was done by a person who was not informed of the purpose of our experiments.

As stated in the last section, the crest time represents a counterpart of the hue, and changes in crest time may be converted into hue shifts in terms of wave-lengths, when the relation illustrated in Fig. 3 are utilized. With the hue matching method, Purdy determined such a hue shift for each wave-length which was caused by raising the intensity from 100 up to 1000 photons. It is of interest to compare our results with Purdy's. In Fig. 6, Purdy's results are represented by open circles, shifts towards longer wave-lengths being plotted upwards, and those towards shorter wave-lengths downwards. Our results which are represented

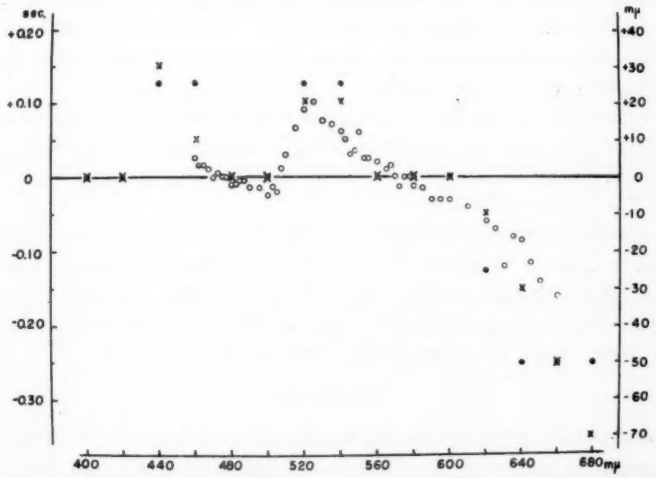

Fig. 6. Comparison of results of present experiment with those of Purdy's experiment. Hue shifts towards longer and shorter wave-lengths are plotted up- and downwards respectively. Purdy's data are represented by open circles. Dots represent changes in crest time and crosses corresponding changes in wave-length.

by crosses refer to hue shifts in terms of wave-length, and those represented by dots to changes in crest time themselves. In both experiments no appreciable color shift can be seen in two regions 480–500 and 560–600 mμ. There are remarkable positive color shifts between 520 and 540 mμ, and the conspicuous negative color shifts in the longer wave-length end of the spectrum. Concerning these aspects, agreement between both experiments is fairly satisfactory, but some discrepancies are found as to other aspects: For example, Purdy found well-localized three invariable points, at 474, 506, and 571 mμ, while in our experiments they were represented by two not so sharply localized regions. This discrepancy may be due to the difference in method. The slight negative shift observed by Purdy between 480 and 500 mμ were not observed in our experiment. As Purdy's experiment was not extended to the violet end of the spectrum, the invariant region found by us between 400 and 420 mμ was missing in his experiment. Some reservation has, however, to be made as to whether the invariant region at the violet and determined by our method really means an invariant one with respect to the sensation of hue, not only because no experiment of hue matching has been carried out for this region, but also because there is something peculiar to this region with respect to the form of the ζ-time curve: In reality, the ζ-time curve obtained for this region consists of two elevations, one at 1.0 sec. and the other at 3.0 sec., although only the latter was measured in the present experiments. It seems that the elevation at 1.0 sec. represents the red component, while that at 3.0 sec. the blue component, and that the hue is determined by the relative magnitudes of these two elevations. Therefore, the invariant with respect to the elevation at 3.0 sec. alone does not justify to assume that there occurs no hue shift in this region.

It is to be noted that the intensity was raised from unit to 100 in our experiment, while the ratio was 1 : 10 in Purdy's experiment. The fact that the positive and negative shifts tend to be greater in our experiment than in Purdy's may be due to this difference.

The present investigation has established that the crest time is a function of hue, and it deserves attention, because there has been no known physiological quantity related directly to the hue. It must further be added that there occurs some color change on transfer from central to peripheral vision, and that the crest time shows the corresponding shift in such cases.[14,15]

SUMMARY

Hue shift with intensity (Bezold-Brücke phenomenon) was investigated from a new angle, by measuring the electrical excitability of the eye after a brief exposure to monochromatic light. The time-course of electrical excitability changed when the wave-length was varied and also when the intensity of the stimulus light of one and the same wave-length was altered. By comparison of

both effects it was possible to express the intensity effect in terms of wavelengths. The results were compared with those obtained by Purdy with the method of hue matching, and satisfactory agreement was found between them.

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Mechanism of Occurrence of Toxemia of Late Pregnancy

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INTRODUCTION

There have been many theories propounded in the past concerning late pregnancy toxemia, as well as the reports on experimental toxemia in animals that were undertaken to enclose them. At present, however, we know of no satisfactory theory on the genesis of the toxemia.

This toxemia is a syndrome, and has various histological changes in many organs.

Accordingly, the present author felt justified in assuming that, if a theory on the toxemia is sound, it must be capable of consistently explaining all these changes, and if an experimental toxemia is a faithful reproduction of the toxemia in human patients, the method used for inducing it or a closely related method should be capable of causing all these pathological symptoms and changes.

The present author and his co-workers have been engaged for these 18 years in searching for such a theory on late pregnancy toxemia and a method of experimentally inducing it, as would fully satisfy the above conditions. We will report in summary on our studies in the following.

EXPERIMENTAL METHODS

a) Stimulation of hypothalamus (K. Kushima)

Taking up the hint furnished by the similarity of many symptoms in toxemia with as many symptoms in diencephalic syndrome we experimented with stimulating the nucleus ventromedialis hypothalamicus of rabbit and succeeded in inducing the toxemia-like changes as listed below. According to the researches of T. Kurotsu[1], the point of an injecting syringe perpendicularly inserted into the cranium of rabbit to the depth of 15.5 mm at a point 1.0–2.0 mm lateralwards from the intersection of the suturae sagittalis and coronalis will reach the N. ventromedialis. Through a syringe thus inserted, we introduced a drop (ca. 0.15 VU) of neurohypophyseal hormone – the hormone that is found particularly

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increased in late pregnancy. Yamada, one of the co-workers, has reported on the increase of this hormone in the diencephalon of pregnant rabbits over the content in that of non-pregnant rabbits, and also found a heightened activity of pitutrin-like substance in the blood sampled from the vena uterina coming from a pregnant uterus. S. Akiyama, one of the co-workers, found the Gomoriphil substance in the hypothalamus of rats markedly increased following transplantation of a slice of placenta beneath the dorsal skin. Accordingly, we may presume that the neurohypophyseal hormone may serve as a stimulant of the hypothalamus.

b) Intravenous injection of placental substances

For this purpose, we used Schneider's thromboplastin[2], Magara's polypeptide[3], Kaku's polysaccaride[4] and also 10 varieties of substances separately extracted from normal and toxemic placentae, respectively, by H. Yoshizaki[5], one of our co-workers (Tab. II). These were intravenously administered to rats.

TABLE I. A List of Symptoms and Changes in Late Pregnancy Toxemia

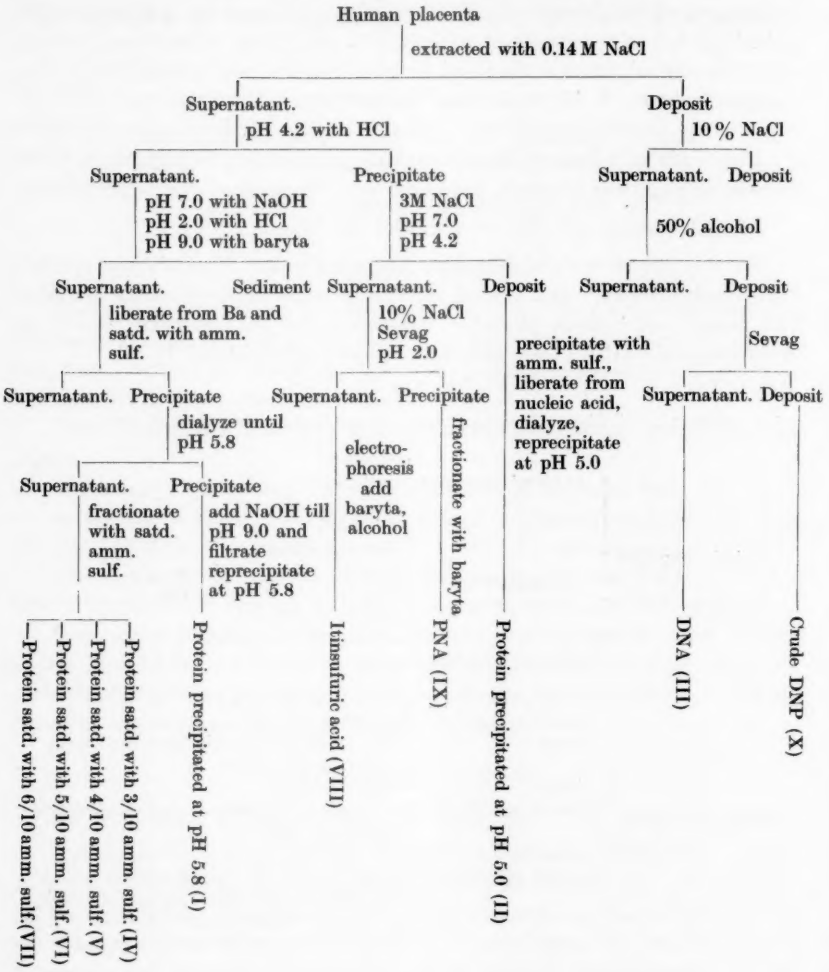
Clinical symptoms	Cardinal symptoms	edema
		proteinuria
		hypertension
	Special toxemias	eclampsia
		abruptio placentae
		toxemic pulmonar edema
		toxemic cerebral apoplexia
		anemia (hydremia)
		retinopathia toxemica
	Chronic (or mixed) toxemia	
Injury of organs	liver	necrosis and bleeding
	kidneys	glomerulo-nephrosis
	heart	cloudy swelling of muscle-fibers and edematous swelling of stroma
	lungs	allergic changes
	suprarenal glands	hypertrophy of reticular zone and cell-degeneration in the zone.
	placenta	brown infarct etc.
	artery of brain	necrosis of the wall of small arteries (primipara). Sclerotic changes of the wall of middle sized arteries (multi-para)
	fetus	damage of liver and kidneys

Our Experimental Toxemia

a) Albuminuria (Co-workers: R. Kikuchi and Y. Kato)

The above method perfected by Kushima was applied in experiment with 18 rabbits, of which 16 excreted 0.2–2.0‰ of albumin in the urine. The albumin content rose to the maximum in 2–4 hrs., then dropped off and became negative

TABLE II. Isolation Process of Placental Components



in 24 hrs. after the injection of neurohypophyseal hormone. When the animals were pretreated with subcutaneous injection of phenobarbital prior to the hormone injection, the albuminuria was perceptibly inhibited. When Ringer's solution was injected instead of neurohypophyseal hormone, the albuminuria was also milder.

The animals were sacrificed while the albuminuria was positive, while no

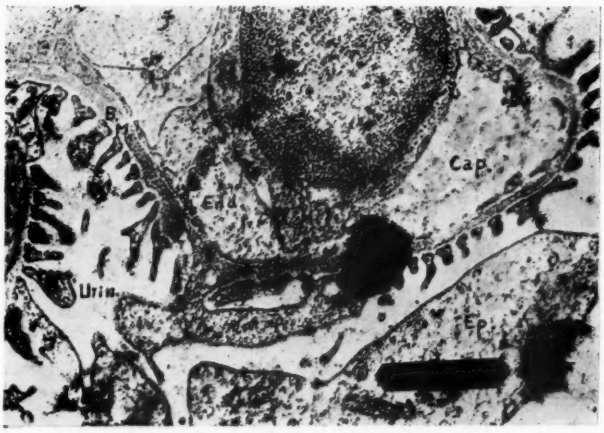


Fig. 1. Glomerular capillary wall of rabbit's kidney without proteinuria.
End: Endothelial cell, Ep: Epithelial cell, B.M.: Basement membrane, R.B.C.: Erythrocytes, Urin: Urinary space Cap: Capillary space

albuminuria was induced and when the albuminuria had disappeared 24 hrs after the hormone injection, and their kidneys were examined under an electron microscope. The basement membrane of the capillaries of the renal glomeruli was found thickened and showing lowered electron density while the albuminuria was positive, but normal in the cases with negative albuminuria (Figs. 1 and 2). That is to say, the fine structure of the glomeruli was found changing in parallel with the grade of albuminuria.

b) Hypertension (Co-worker: F. Narita)

Assali claims that toxemic hypertension is induced by humoral factors and cannot be suppressed with sympathetic blockers, but clinical experiences have revealed that such blockers are effective in relieving hypertension in some toxemic cases and not in the other, that is, some of the cases have nervous hypertonia and the others humoral hypertension. In the animals with experimental toxemia induced by Kushima's method, a rise of blood pressume, by 32 mm Hg at the maximum, was always present, though the rate of rise showed individual difference. This hypertension seems to be due to neural factors.

c) Edema (Co-workers: M. Tanaka, F. Shigeno, K. Hagane and R. Komukai)

With the aim of testing the water affinity of the skin, reflecting the edematous tendency, urticae were formed by intradermal injection of physiological saline and the time of absorption was measured. The absorption time was found

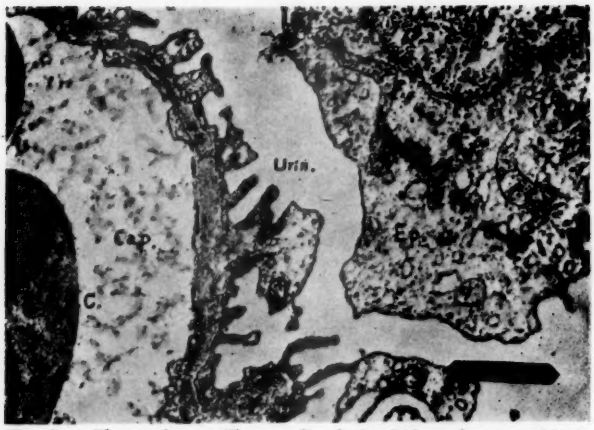


Fig. 2-a Glomerular capillary wall of the kidney from a rabbit with marked proteinuria, two hours after the injection.

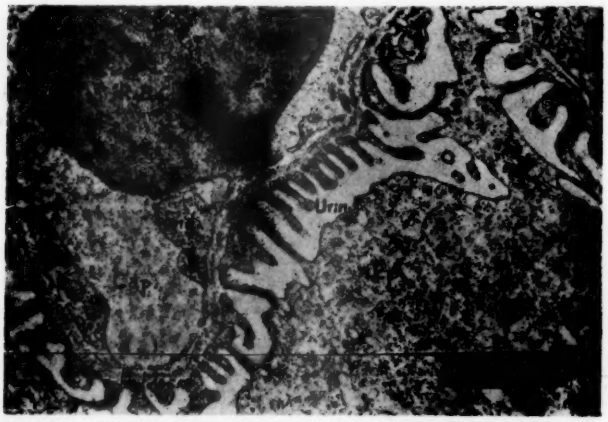


Fig. 2-b Glomerular capillary wall of the kidney from a rabbit twenty-four hours after the injection. (When proteinuria disappeared)
(The scale in each figure indicates 1 μ length.)

shortened 60 min. after stimulation of the hypothalamus, indicating a strengthened tendency to edema.

By measuring the potential generated by insertion of chlorine electrodes beneath the sking of rabbit with a vacuum potentiometer, the potential proportionate to the Cl concentration was found gradually rising after stimulation of hypothalamus. As it is presumable that the Cl content in tissues migrates

in combination with sodium, this finding also suggests a strengthening of the tendency to edema.

d) Eclampsia (Co-worker : H. Tani)

No convulsion could be induced by a single injection of a drop of neurohypophyseal hormone into the hypothalamus, but when the injection was daily repeated, eclampsia-like convulsions were caused after the 3rd or 4th round in about half of the cases.

e) Abruptio placentae (Co-worker : C. Nakazato)

A review of the past reports on successful experiments of inducing apoplexia uteroplacentae in animals raises doubts about the true nature of the results whether an abruptio or an abortion is represented in most cases. Therefore, we sought for some symptoms besides ablation of the placenta characterizing this trouble in 10 human uteri of uteroplacental apoplexy cases, and found bleeding and edema, sometimes rupture of small veins, in the myometrium to be specific to such apoplexy. The fibrinous necrosis of the artery walls and fibrinoid thrombosis as cited in the past reports were indeed observed in our study too, but we did not think it probable that these are the cause of the trouble.

When the nervus splanchnicus of pregnant rabbits was electrically stimulated, in accordance with the method of experiment by Couvelaire[6], edema was caused in the myometrium, but no hemorrhage. Therefore, we inferred that there must be some additional factor for actual occurrence of abruptio and considered the factor of sclerosis of the arteries, as frequently observed in human abruptio placentae, in addition to the direct cause. Thus, 0.5 cc each of 0.1% epinephrine was subcutaneously injected for 10 consecutive days and then the N. splanchnicus was stimulated. This caused occurrence of obvious abruptio-like changes. When injection of small doses of neurohypophyseal hormone into the nucleus ventromedialis hypothalamicus is kept up for many days, for the purpose of stimulating the center of sympathetic nervous system, similar abruptio-like changes were induced. In this case too, a preceding injection of epinephrine continued for some days would intensify the changes induced.

From such experimental results, it may be deduced that abruptio placentae is caused by excessive stimulation of the sympathetic nerve, that is, it is a Reilly's phenomenon. Such an excessive stimulation may be caused by an increase of the neurohypophyseal hormone content in the diencephalon or a release of some placental substance, as stated below, into the blood. Abruptio may follow a mere stimulation of the nerve center, if it is strong enough, but if arteriosclerosis preexists, it may be more readily induced.

f) Toxemic pulmonary edema (Co-worker : J. Nishi)

Only Vaglio[7] has reported on successful production of experimental

toxemic pulmonary edema. He says that intravenous injection of epinephrine preceded by injection of gonadotrophin induces such a change, but our follow-up experiment failed in obtaining the result. We injected 0.14–0.25 VU per kg of body weight of neurohypophyseal hormone into the cisterna of rabbits and 0.085–0.25 mg/kg of epinephrine 5–10 min. after it, and severe pulmonary edema could be induced. Use of Ringer's solution instead of the hormone or mere injection of the hormone into the cisterna alone caused no pulmorary edema. It has been ascertained that a part of the neurohypophyseal hormone secreted in the diencephalon is transported to the third ventricle. This means that when a hypersecretion of the hormone occurs in the hypothalamus, the content of the homone in the ventricle also increases, and a preparatory stage for pulmonary edema to occur is established. Injection of epinephrine may be assumed to cause an irritation of the sympathetic nervous system or quasi-emergency reaction. The low frequency of actual occurrence of toxemic pulmonary edema is probably due to the rarity of coexistence of such a preparatory stage, a state like that following epinephrine injection.

g) Toxemic cerebral bleeding (Co-worker : H. Saito)

Most of cerebral bleeding in toxemia is associated with eclampsia. Histological examination of cerebral arteries of the cases killed by eclampsia plus cerebral hemorrhage, revealed that in the primiparae, the cerebral arterioles were subject to necrosis, while in the multiparae, hypertensive sclerotic changes were the leading findings in the brain arteries. Intravenous injection of cardiazol caused vehement convulsion but no change in the cerebral blood vessels in rabbits. Rabbits with eclampsia-like convulsions induced by daily injection of neurohypophyseal hormone in the nucleus ventromedialis hypothalamicus for sufficiently many days were found to show changes in the cerebral arterioles similar to those observed in human primiparous eclampsia cases. This experimental method, however, failed in causing cerebral vascular changes as observed in multipara eclampsia cases.

h) Changes in the eye fundus (Co-woker : M. Yamada)

The eye fundus, or the retina, of the toxemic cases is subject to contraction of the blood vessels and edema. Only spasms of the retinal blood vessels immediately followed stimulation of the hypothalamus, but in 20 min. or so, the papilla began to swell, obscuring their boundaries, and some other findings indicative of fundus edema also appeared.

i) Functional and histological changes of the liver (Co-worker : K. Nagai)

Upon testing the liver function of diencephalon-stimulated rabbits by bromesulfalein test, the function was found mildly or moderately impaired. Changes

reminding of eclamptic liver were observed in the rabbits with repeatedly stimulated midbrain.

j) Histological changes of the kidney (Co-worker: R. Kikuchi)

Findings indicative of glomerulonephrosis were found mildly in the kidneys of rabbits stimulated hypothalamus.

k) Ditto of the heart (Co-worker: M. Maeno)

Autopsy of cases who had toxemia in life revealed advanced cloudy swelling of the cardiac muscles, atrophy of the muscle fibers and edematous swelling of the stroma. In the animals with experimental toxemia induced by our method, such cardiac changes were found more intense than in animals with experimental toxemia induced by any other method.

l) Ditto of the adrenal cortex (Co-worker: K. Izaka)

Histological examination of the adrenal cortex of the pathologically autopsied cases with and without toxemia in comparison showed that hypertrophy of the glomerular zone, degeneration of the cells and diminution of lipids in that zone were the characteristic findings in the toxemic cases. Such changes were frankly observable in the adrenal cortex of our rabbits with stimulated hypothalamus.

m) Ditto of the liver and the kidneys of the fetus (Co-worker: S. Miyauchi)

It is a well-known fact that changes similar to the toxemic changes observed in the maternal liver and kidneys are reproduced in the same organ of the newborn child.

When 2 or 3 injections of small dosis of neurohypophyseal hormone are administered to pregnant rabbits into the hypothalamus, changes suggestive of eclamptia liver and glomerulonephrosis were observed in the fetus.

n) Allergic changes of tissues (Co-worker: K. Nagai and K. Sasano)

Many authors claim the nature of toxemia to be allergic, because of the presence of allergic changes in various organs in the affected cases as the ground of their claim. In our animals with experimental toxemia induced by means of stimulation of the hypothalamus, there is no chance for occurrence of antigen-antibody reaction. Nonetheless, the liver of rabbits with neurohypophyseal hormone instilled in the hypothalamus showed allergic changes, including edematous swelling of the connective tissue in the Glisson's capsules, infiltration of round cells and histiocyte-like cells into them, thickening and swelling of the arterial walls, proliferation of the nuclei in the arterial intima and hematostasis and fibrin-thrombi in the portal vein. Such allergic changes were present also in

the lung. Accordingly, we may affirm the resemblance of our experimental toxemia to the human toxemia in respect of allergic findings too.

o) Histological and electron-microscopic changes in the placenta (Co-worker: (K. Noda, I. Noji and K. Aneha)

Our investigation revealed that brown infarct is an item specific to human toxemia. White infarct, syncitium-cell nodes, mixed figure of anaemic and hyperaemic villi, fibrin-thrombi in the chorion, capillarectasia or capillary contraction, haematomata in the intervillous spaces, fibrinoid deposits on the villi or the decidua, hemorrhage in the decidua and the placental stroma, necrosis and hypertrophy of the vascular walls, infiltration of small round cells into the stroma and edematous change of the placental septa could be more frequently detected in toxemic than in normal placenta. Such toxemic placental changes were evoked very perceptibly upon stimulation of the hypothalamus. Considering such findings, we may infer that, instead of primary occurrence of placental changes causing toxemia, anomalies are first induced in the midbrain, involving occurrence of changes in the placenta, which in turn entail production of the substances that partake in the genesis of toxemia.

It has been the accepted opinion that infarct of the placenta is caused by necrosis due to disorders in the blood flow to the peripheral part of the blood vessels following spasms of blood vessels in the villi. But upon closer examination, it was found that sometimes the infarcted villi contain fresh erythrocytes, and sometimes are covered by deposited fibrin. These findings seem to suggest that the anomalies produced in the maternal-side vascular system entails changes in the blood filling the intervillous spaces, causing precipitation of fibrin on the villi, which are thereby hampered in their alimentary ingestion and finally fall a prey to infarct.

Upon examination of normal placenta and placentae sampled from acute and chronic (mixed) toxemia cases under an electron microscope, changes specific to toxemic placentae were detected. Whereas only a small number of endoplasmic reticula could be found in the syncytial cells of normal placentae, innumerable endoplasmic reticula could be observed in those of toxemic placentae, giving a honeycomb-like appearance to the cells. In acute toxemia, the vacuoles are of nearly uniform size, but in chronic toxemia these were of varying sizes and the whole appearance reminded of a cystoglandular hyperplasia of the endometrium (Figs. 3. 4. 5). Similar findings were observable when intermittent anoxia was induced in the placenta or the hypothalamus was stimulated in our experimental animals.

p) Other changes

Anaemia in toxemia and chronic toxemia could not be induced by our experimental method of stimulating the hypothalamus.

Fig. 3. A chorionic epithelium-cell in a placenta from normal gravida of 10 months of pregnancy. (× 5,000)

IV.	intervillous space	Sy:	syncytium-cell layer
BM:	basement membrane	La:	Langhans' cell
S:	stroma cell	En:	endothel cell of capillary in fetal placenta
MV:	microvilli	M:	mitochondria
ER:	endoplasmic reticulum	N:	nucleus
L:	cavity of capllary in fetal placenta		

Toxemia and Placental Components

The placental components isolated from normal human placentae consisting in Magara's substance (polypeptide), Kaku's substance (polysaccharide) and thromboplastin, and 10 substances (Tohoku substances) isolated from normal and toxemic placentae were experimentally tested.

a) Magara's substance

Pregnant rabbits were injected with 20 mg each of PS fraction of Magara's substances. The urinary protein remained negative following the injection in the animals with originally negative protein in urine, but in the animals showing positive urinary protein before the injection, the value was found risen very slightly after it.

Following the injection, the blood pressure always rose, by 32 mmHg at the maximum, but this rise could be totally inhibited by administration of phenobarbital.

This PS substance was next only to hypothalamus stimulation in the effect

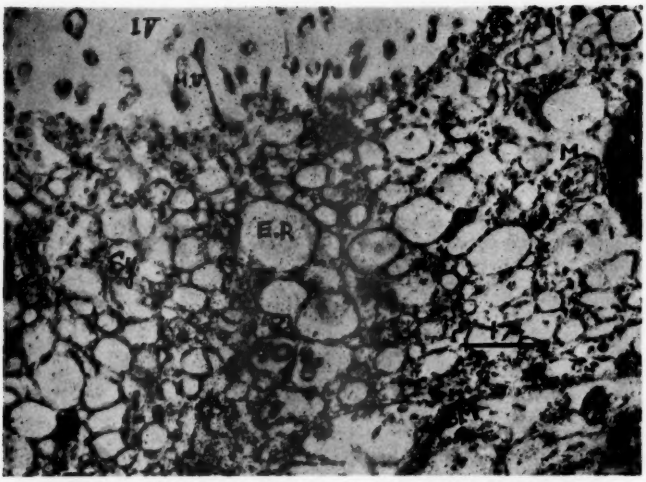


Fig. 4. Chorionic epithelium-cell of a mature placenta from acute toxemic gravida. (× 5,000)

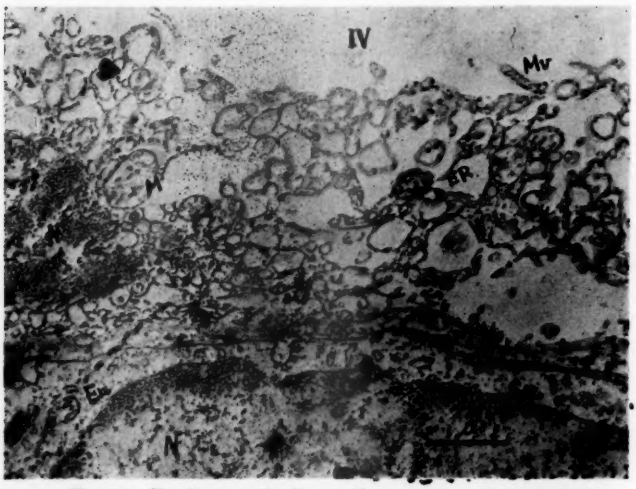


Fig. 5. Chorionic epithelium-cell in mature placenta from the patient of mixed (chronic) toxemia.

of strengthening the proneness to edema.

PS injection facilitated subsequent occurrence of Reilly's phenomenon and consequently changes resembling apoplexia uteroplacentae were sometimes

observed.

In the PS-injected rabbits, the liver, the kidneys, the heart, the adrenal cortex and the placenta were found showing very prominent toxemia-like histological changes, but the injection could not induce eclampsia-like convulsions or pulmonary edema.

b) Kaku's substance

Pregnant rabbits were intravenously injected with 100–200 mg of Kaku's substance (KPS). It showed as little effect of intensifying proteinuria as Magara's substance, that is, nearly none. The effect in promoting water retention was weaker than with hypothalamus stimulation or Magara's substance. The injection caused mild toxemia-like histological changes in the liver, the kidneys and the heart of the animals and the liver and the kidneys of their fetus, but no histological changes in the placenta and the adrenal cortex, no convulsion and no pulmonary edema at all.

c) Thromboplastin

Sechneider opines that thromboplastin is the cause of toxemia. Pregnant rabbits were intravenously injected with 5 cc of 1 : 200 diluted supernatant obtained by centrifuging 0.1 g of thromboplastin extracted from rabbit brain with aceton and mixed with 2 cc of physiological saline. This injection caused no toxemic symptom.

d) Tohoku substances (Co-worker: H. Yoshizaki, K. Noda and R. Hashimoto)

The underlisted 10 different substances were separately extracted from normal and toxemic placentae by the isolation method shown in Tab. II.

The effect on blood pressure and the antidiuretic action were the strongest with Comp. IV from toxemic placentae and Comp. I from normal and toxemic

Table III. Tohoku Substances

Component	I :	Protein which is insoluble in water at pH 5.8.
Comp.	II :	Protein which is insoluble in water at pH 5.0.
Comp.	III :	DNA.
Comp.	IV :	Protein which is precipitable at 3/10 saturation of its solution with ammonium sulfate.
Comp.	V :	Protein which is precipitable at 4/10 saturation of its solution with amm. sulf.
Comp.	VI :	Protein which is precipitable at 5/10 saturation of its solution with amm. sulf.
Comp.	VII :	Protein which is precipitable at 6/10 saturation of its solution with amm. sulf.
Comp.	VIII :	Itinsulfuric acid.
Comp.	IX :	PNA.
Comp.	X :	Crude DNP.

placentae. Comp. IV from toxemic placentae only showed a barely perceptible effect of aggravating proteinuria. The histological changes following injection of Comp. IV were much more intense for experimentally inducing toxemia. For the other findings, vide Tab. IV.

TABLE IV. Effect of Tohoku Substances in Causing Toxemic Changes

Comp. No.	I		II		III		IV		V		VI		VII		VIII		IX		X	
From normal or toxemic placenta	N*	T**	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
Hypertensive	++	++	+	+	+	+	+	++	+	+	−	−	+	++	−	−	−	+	−	+
Antidiuretic	+	+	−	+	−	−	+	+	+	+	−	−	+	+	−	−	−	−	−	−
Causing Proteinuria	±	±	−	±	−	−	+	+	−	−	−	−	−	±	−	−	−	−	−	−
Causing change in																				
Liver	−	±	−	+	−	−	+	++	−	−	−	−	−	−	−	−	−	+	±	+
Kidneys	−	−	−	−	−	−	+	++	+	+	−	−	−	−	−	−	+	+	−	−
Heart	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	+
Adrenal gland	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Placenta	−	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−	−	−

Note: *N .... normal placenta
**T .... toxemic placenta

The results show that generally a component extracted from toxemic placenta has stronger effect than the same component extracted from normal placenta, except the effect on blood pressure in Comps. II and V, and that the different components exert effect on different organs.

e) Corticoids in placenta (Co-worker: T. Adachi, H. Takikawa and K. Saito)

No DOC was detected in the placenta but some other chemocorticoids could be detected by paper chromatography, as shown in Tab. V.

The anemia in toxemic cases consists mainly in hydremia mixed with slight genuine anemia. When rabbits were injected with mineral-corticoid,

TABLE V. Chemocorticoids in μg (per placenta)

Placenta	Glucocorticoids			Mineralocorticoids**
	Tetra-h.-fr.*	Comp. F.	Comp. E.	
Normal (spont. delivery)	32.2	8.7	60	17.5
Normal (cesarean section)	45	12.5	50	15
Toxemic (spont. delivery)	40	8.3	66.6	30

Note: * Tetra-hydro-fraction, giving spot more polar to that of Compound F.
** Giving spot between Compound B and DOC.

gonadotrophin, cortisone (Compound E) or posterior pituitary hormone, a change resembling toxemic anemia was induced in them.

f) Pituitrin-like substance and amines from placenta (Co-worker: K. Honda and T. Kadowaki)

In the human placenta, we could demonstrate 10–18 mU/g of oxytocin-like substance. This value was somewhat higher than the content of the substance in the maternal serum. Guggisberg[7] and Fellner[8] have already reported on the vassopressin-like substance extracted from the placenta, and we also could comfirm the strong pituitrin-like action of it.

The content of 5–hydroxytryptamin (serotonin) in the placenta was 0.12 μg/g and that in blood was 0.08 μg/ml.

The other contents of the placenta were nor-adrenalin, guanidine, tilamin and some substances not to be identified. Substances mentioned above seemed to cause some types of hypertonia as well as anemia.

Toxemia and Corticoids

a) Glucocorticoids (Co-worker: H. Kawahara and H. Takikawa)

The past reports claim that the urinary 17-OH-corticoid is reduced in toxemia. The results of bioassay on the subject by Kawahara confirmed this claim. When, however, Takikawa measured the urinary content of chemocorticoids in toxemia, Compound F and Compound E as well as the total 17-OH-CS were found larger than in normal pregnancy.

b) Mineralocorticoids (Co-workers: T. Adachi and H. Takikawa)

DOC was detected neither in the urine nor in the blood (500 cc) of toxemic patients. A mineralocorticoid forming a spot between those of DOC and Compound B in paper chromatography (Bush C system) was found increased in toxemia., increasing and decreasing parallel with the grade of the trouble. Aldosterone was found increased in the gravidae with severe edema.

Toxemia and Renin

Y. Kikuchi, one of the co-workers, extracted renin by an improved method devised by judicious combination of the processes of Yoshida[9] and Landis[10]. He then measured the renin content in serum of 20 toxemic patients and proved its existence in 10 cases among the hypertensive toxemias. A renin-like substance was isolated from the placenta and the lung of animals in which anoxia had been induced.

Of the types of toxemia, the mixed type (the authors are used to call it "chronic toxemia") represents the disorder with long-persistent toxemic symptoms after delivery. These persistent symptoms consist in most part of hypertension

or hypertension plus proteinuria and but rarely of proteinuria alone. Thus, chronic toxemia nearly always shows the symptom of hypertension and accompanies sclerotic changes in the small blood vessels.

In the above-described experimental toxemia, we could not induce disturbance resembling chronic toxemia. Masson *et al*[11)-12)] report that injection of renin plus DOC or renin puls glucocorticoid induces vascular sclerosis in animals. Our follow-up experiments brought about similar results: When rats were injected subcutaneously with hog renin, in combination with 3 mg of DOC, with 1.5 mg of cortisone or 1.5 mg of hydrocortisone, over 14 days in succession, sclerosis was induced in the renal blood vessels. The same result was obtained when the renin-like substance extracted from the placenta was applied in the same manner instead of hog renin.

These results suggest that chronic toxemia is induced when renin or the renin-like substance from the placenta comes into reaction with the mineralo- and gluco-corticoids serected from the adrenal glands and the placenta over considerable duration. In human cases also, it has been observed that chronic toxemia is apt to occur when toxemic hypertension has persisted for more than 2 weeks during pregnancy.

COMMENT

Late pregnancy toxemia is a syndrom. It would be more convenient for us if the genetic factor of this syndrome is single, but if we start to search for such a single cause from the outset, it may lead to erroneous conclusions. It seems, however, very probable that one principal cause is responsible for the major parts of the symptoms.

After many years of research work and observation of many toxemia cases, we have been led to infer that the cause and the mechanism of pathogenesis of toxemia are something as summarized in the following:

When the influence of the physiological phenomenon of pregnancy goes beyond the capacity of the pregnant to stand, a disturbance occurs in the function of the hypothalamus. This disturbance is probably occasioned by stimulated neurosecretion and placental products, and amplified by emotional uneasiness *etc.* The dysfunction of the hypothalamus, besides causing proteinuria, edema, hypertension, eclampsia, preparatory tendency to pulmonary edema, change of the eye fundus and toxemic histological changes in the liver, the kidneys, the heart, the adrenal glands and the fetus, also causes change in the nature of the blood in the intervillous spaces, to form deposits of fibrin on the villi which in turn entails anoxia of the fetal placenta, thus completing the specific changes of toxemic placenta. Many kinds of protein are then released from the placenta, which causes edema, hypertension, abruptio placentae, toxemic anemia, and toxemic changes

in the maternal liver, adrenal glands, heart and kidneys and in the fetus. Different kinds of protein thus released cause troubles in different organs. The proteins released from the placenta constitute noxa also to the placenta itself and further aggravate the toxemia. Amines are also released from the placenta and beget hypertension.

The adrenocortical hormones, besides causing edema and toxemic anemia, cause chronic (mixed) toxemia by coming into reaction with renin or the renin-like substance produced in the placenta.

Chronic toxemia induces occurrence of abruptio placentae and eclamptic cerebral hemorrhage in multipara. The Reilly's phenomena due to release of adrenalin seem to play some part in the occurrence of toxemic pulmonary edema and abruptio placentae. The above process is schematized in Fig. 6.

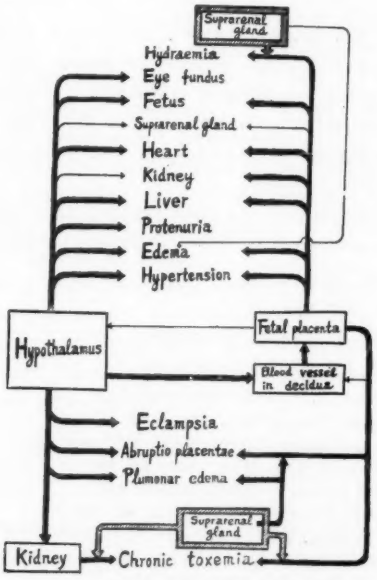


Fig. 6. Mechanism of Pathogenesis of Late Pregnancy Toxemia.

CONCLUSION

Basing ourselves on the results of a long series of experiments, the present author has made inferences on the probable genesis of late pregnancy toxemia as summarized as follows:

1) If the influence of normal pregnancy gets beyond the capacity of the pregnant to withstand, a disturbance is induced in the function of her hypothalamus.

2) The dysfunction of the hypothalamus directly causes edema, proteinuria, hypertension, eclampsia, retinal changes, histological changes in some maternal as well as fetal organs, and toxemic changes in the fetal placenta. Many kinds of protein are released from the affected placenta.

3) These proteins from the placenta in turn cause edema, hypertension, histological changes in some organs and the fetus, as well as anomalies in the placenta itself. Besides, the placenta secretes amines effective in causing hypertension and mineralo- and gluco-corticoids causing toxemic anemia.

4) The hormones secreted by the adrenal glands, in reaction with the corticoids from the placenta, cause edema and hydremia (toxic anemia).

5) Renin and the renin-like substance released from the placenta, when coming in reaction over long duration with corticoids, cause chronic or mixed toxemia.

6) The Reilly's phenomenon caused by release of adrenalin is responsible for the occurrence of toxemic pulmonary edema and abruptio placentae.

7) Thus, toxemia is a syndrome of which the dysfunction of the hypothalamus and the placental substances released thereby is the primary cause and the adrenocortical hormones, renin, adrenalin and neurohypophyseal hormones play the part of secondary causes.

8) From the above findings, we may conclude that late pregnancy toxemia may not be called "a genuine toxemia" but should be called "a dysadaptation syndrome of pregnancy".

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Tohoku J. Exper. Med., 1961, 74, 105-112

Quantitative Determination of Metadrenaline and Normetadrenaline in Human Urine

By

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(Received for publication, December 12, 1960)

Since Armstrong *et al.*[1] found 3-methoxy-4-hydroxymandelic acid (MHMA) in normal human urine as an end product of catecholamines in 1957, the metabolism of catecholamines have been studied by several investigators. Soon afterward, Axelrod and his coworkers[2][3][4][5] found 3-methoxycatecholamines in the urine of rats. LaBrosse *et al.*[6] reported in 1960 that normal human urine contained methoxycatecholamines, but their concentrations were not measured. LaBrosse *et al.*[7] found 47~59% of radioacitivity in a methoxycatecholamine fraction of human urine obtained after infusion of ^{3}H-labeled adrenaline, and Goodall[8] found about 34% of it.

These results seem to show that methoxycatecholamines are present in normal human urine, as Kirschner *et al.*[9] mentioned previously. However, their concentrations in normal human urine have never been quantitatively determined up to now. The present paper deals with the method to determine the urinary methoxycatecholamines excreted by man.

EXPERIMENTS AND COMMENTS

Reagents

a) *Buffer for high-voltage paperelectrophoresis*: Pyridine-acetic acid-water (10 : 100 : 890 v/v, pH 3.6).

b) *Diazo-reagent according to von Studnitz*[10]

(1) 0.1 g. of p-nitroaniline dissolved in 2 ml. of conc. HCl and diluted with distilled water to 100 ml.

(2) 0.2% aqueous solution of sodium nitrite.

(3) 10% aqueous solution of potassium carbonate.

All these solutions were kept in a refrigerator. Just before use, one volume of (1), one volume of (2) and two volumes of (3) were mixed. The mixture must

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be used within 120 seconds.

c) *Eluent*: Mixture of methanol and 2% sodium carbonate (2 : 1 v/v).

Procedure

a) *Material*: The urine which was collected for 24 hours in a bottle containing about 10 ml. of conc. HCl was used.

b) *Extraction*: 5~10 ml. of the acidified urine was adjusted to pH 1.0 and hydrolysed in a boiling water bath for 1 hour. The hydrolysate was cooled in tap water, adjusted to pH 10.0 with 20% NaOH solution, saturated with NaCl, mixed with 1 ml. of 0.5 M borate buffer (pH 10.0) and finally readjusted to pH 10.0 (indicator paper). This sample was extracted 3 times with 5 volumes of ethyl acetate by shaking vigorously for 5 minutes. Following centrifugation, the organic phase was collected and distilled *in vacuo* to dryness at a temperature below 60°C. The residue was dissolved in 2 ml. of methanol and transferred to a small glass tube. This procedure was repeated 3 times. The methanol extract was distilled under reduced pressure to dryness as above. The residue was quantitatively taken up in 0.2 ml. of methanol and again in 0.1 ml. of the same solvent for plotting.

c) *Isolation of metadrenaline* (*M*) *and normetadrenaline* (*NM*) *by high-voltage paperelectrophoresis*: The apparatus used was of a horizontal form, as shown in Fig. 1. The sample (0.3 ml.)was plotted linearly on Toyo Roshi filter paper No. 51 (5×60 cm.) at a distance of 8.5 cm. from the anode end and subjected to high-voltage paperelectrophoresis using hexane as refrigerating insulator. With an operating voltage of 2500~3000 volts and a current of 10 mA. between the electrodes, M and NM migrated about 40 cm. towards the cathode in 2 hours. Fig. 2 shows the schema of electrophoretic pattern of the urinary extract.

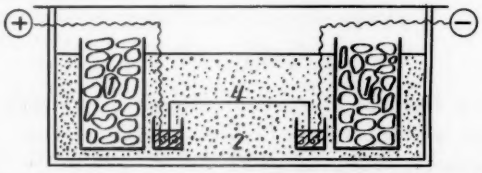

Fig. 1. Apparatus for high-voltage paperelectrophoresis.
1, chopped ice; 2, hexane as refrigerating insulater;
3, developing solvent; 4, filter paper; 5, electrodes.

d) *Measurement of extinction and calculation*: After removal from the apparatus, the filter paper was dried for 20 minutes in a thermostat at 60°C. It was then indicated sufficiently by spraying diazotized p-nitroaniline as mentioned before and dried again. The violet bands corresponding to M and NM were cut out into pieces and the stained area was eluted with 4 ml. of alkaline methanol

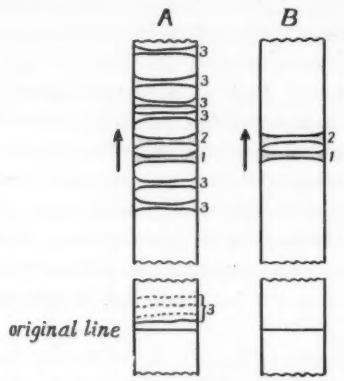

Fig. 2. Schema of electrophoretic pattern.
A. urinary extract; B. reference runs of authentic M and NM; 1, M; 2, NM; 3, unknown substances.

respectively. At the same time a slip of the filter paper of corresponding size was subjected to the same procedure and used as a blank. After standing for more than 1 hour with occasional shaking at room temperature, the intensity of the colour was measured spectrophotometrically at 520 mμ. (Beckman quartz spectrophotometer Model DU). The concentrations of M and NM in the urinary extracts were then calculated from the standard curves for the authentic M* and NM* aqueous solution.

Comments on the Method

a) *Extraction*: The pH of the aqueous solution of M or NM was brought up to 10.0 with 20% NaOH solution, and the solution was saturated with NaCl. M or NM in the solution was then extracted 3 times with 5 volumes of ethyl acetate. Each extract was analysed separately, the results of which are given in TABLE I. Ethyl acetate did not extract any amount of inorganic salts, which, if present, would have disturbed the further procedures seriously. Alcohols (n-butanol, iso-butanol and iso-amylalcohol were examined) extracted inorganic

TABLE I. Extraction with Ethyl Acetate

	Metadrenaline (%)	Normetadrenaline (%)
1st extraction	67.9	64.3
2nd.	17.4	22.4
3rd.	9.3	3.8
Total	94.6	90.5

salts under this condition. Ethyl ether and ethylene dichloride were less effective than ethyl acetate. Purification of methoxycatecholamines with ion-exchange resin was also unsuccessful.

b) *Paperelectrophoresis*: High-voltage paperelectrophoresis described above proved most suitable to separate M and NM not only from the other substances in the extract but also from each other. Moreover it takes only 2 hours. Paperchromatography using several solvent systems could not separate these substances so completely as electrophoresis, and it was fairly time consuming.

c) *Diazo-reaction*: When the amounts of M or NM on filter paper were large, the reaction was apt to be incomplete unless a sufficient quantity of the reagent was sprayed, so that the reagent should be sprayed on both sides of the paper.

d) *Elution with alkaline methanol*: After drying the sprayed filter paper, elution must be started within 1 hour. When more than 1 hour elasped, the

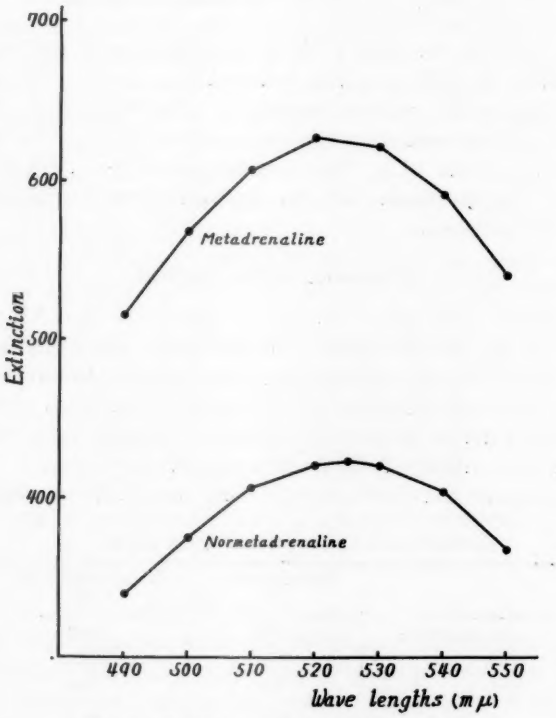

Fig. 3. Extinction curves of M and NM.

whole paper became gradually pink, resulting in higher blank readings, and accordingly, in lower estimated values. The elution was found to complete in about an hour, since the extinction of the eluates was unchanged after 1 hour up to 24 hours. The extinction curves of these eluates are given in Fig. 3.

e) *Hydrolysis of urine*: When the urine was not hydrolysed, the estimated values were much lower than when hydrolysed. In Fig. 4 is given the relation between time of hydrolysis at pH 1.0 and the estimated values. Though an hour is not optimum for hydrolysis, it is conveniently chosen in our laboratory. When the acid-hydrolysis was performed at pH 0.0, the yield was much lower than at pH 1.0. It was found that incubation of urine with bacterial β-glucuronidase caused no increase in M and NM.

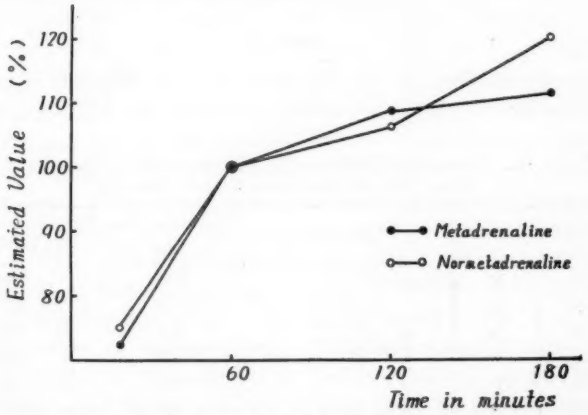

Fig. 4. Relation between time of hydrolysis and estimated values. Value of 60 minutes is expressed as 100%.

f) *Blank*: When the size of filter paper for the blank was not equal to that of M and NM, a large error might be introduced into the estimation, because

Table II. Over-all Recovery

Exp. No.	Recovery (%)	
	Metadrenaline	Normetadrenaline
1	83.7	83.9
2	90.2	75.4
3	87.2	75.4
4	98.8	65.6
5	80.6	83.3
6	100.0	85.8
Mean	90.1	78.3

the reading of the blank was considerably high. The slip of the blank should be cut in the very same size as that of M and NM.

g) *Over-all recovery*: To evaluate the over-all recovery, known amounts of M and NM were added to hydrolysed urine, which was then estimated with the method described here. Satisfactory results were obtained (TABLE II).

TABLE III. Reproducibility

No. of determination	Metadrenaline (μg./24 hr.)	Normetadrenaline (μg./24 hr.)
1	243	215
2	236	254
3	245	259
4	256	245
5	243	227
Mean±S.D.	245±6.5	240±16.6
S.D. (%)	2.65	6.93

TABLE IV. Amounts of Metadrenaline and Normetadrenaline in 24 hours Urine from Patients with Various Diseases.

Subject	Sex	Disease	Metadrenaline (μg.)	Normetadrenaline (μg.)
K. Y.	F.	Healthy	246	239
Y. H.	M.	〃	256	245
A. G.	M.	〃	254	186
K. Y.	M.	〃	172	135
T. K.	F.	〃	257	97
T. C.	F.	〃	125	147
H. S.	M.	〃	225	230
K. M.	M.	〃	104	127
K. O.	M.	Essential hypertension	153	156
J. K.	M.	〃	211	143
S. O.	M.	〃	255	203
S. S.	M.	〃	199	96
S. S.	F.	〃	120	119
M. T.	M.	〃	105	160
S. S.	M.	Graves' disease	143	230
M. H.	M.	〃	128	201
M. S.	F.	〃	182	127
T. S.	M.	Parkinsonism	192	209
G. W.	M.	Primary aldosteronism	231	132
S. I.	M.	Hysteria	416	449
A. I.	M.	Phaeochromocytoma	31,400	5,570
J. N.	F.	〃	7,700	8,270
T. M.	M.	〃	14,200	4,760

h) *Reproducibility*: A urine sample was estimated repeatedly to test reproducibility (TABLE III). Errors ranging about $\pm 7\%$ were inevitable.

i) *Stability of M and NM*: M and NM in acidified urine were found stable at least for a week, even when the urine was kept at room temperature. No change was observed either for 2 weeks when the methanol extract was stored in a refrigerator. Again no loss took place during paperchromatography by use of such strong alkaline solvent as isopropanol-ammonia-water. These facts indicate that M and NM are considerably stable.

j) *Normal values*: M and NM in 23 urine samples from normal subjects and various patients, including 3 cases of phaeochromocytoma have been determined (TABLE IV). The amounts of M and NM excreted from normal subjects during 24 hours may be considered 100~300 μg., respectively.

DISCUSSION

In recent literature there have been many investigations on M and NM, but they mostly dealt with the urine of rats[2)3)5)11)] which excrete relatively large amounts of M and NM, or with isotope tracer[7)8)] of these compounds which can be readily detected by their radioactivity. Determination of M and NM in human urine was performed only in patients with phaeochromocytoma.[12)] It has never been known how much of these substances are excreted in normal human urine. The method presented here is relatively simple, reproducible and sensitive enough to estimate such low concentrations of these metabolites as are excreted in normal human urine. The method would be useful in the studies on the metabolism of catecholamines. With this method, daily urinary excretion of both M and NM from normal subjects was estimated as 100~300 μg. But the values might be by some 30 % lower than the actual amounts, since 10~15% of conjugated M and NM was not yet hydrolysed under the condition employed here, and 10~20% of M and NM was lost during the procedures of determination.

Patients with phaecohromocytoma excrete extraordinary quantities of these methoxycatecholamines. Therefore the diagnosis of this disease can be made by the estimation of these metabolites as well as by the determination of catecholamines.

It is a strange fact that the amount of urinary M is about the same as that of NM, while urinary adrenaline is far less than noradrenaline. This might be explained as follows: NM is formed in the body in larger quantity than M, but the speed in oxidative deamination of the former is higher than the latter. This explanation is supported by some evidences[8)13)] indicating that NM is more easily subjected to oxidative deamination by monoamine oxidase than M.

SUMMARY

1. A method of measuring the concentrations of M and NM in human urine was described.

2. The normal human excretes daily 100~300 μg. of M and almost the same amount of NM in the urine. The concentrations of M and NM in the urine from patients with Graves' disease, essential hypertension, primary aldosteronism and Parkinsonism were within the normal ragne.

3. The concentrations of M and NM in the urine from patients with phaeochromocytoma were abnormally high.

Acknowledgement

The authors are indebted to Prof. Dr. T. Torikai for giving the opportunity of carring out this research, and his continued direction.

* We are greatly indebted to Prof. Julius Axelrod for giving us metadrenaline and normetadrenaline.

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Addendum

While this report was in press, we knew von Studnitz (Studnitz, W. v., Clin. Chim. Acta, 1960, **5**, 447) had reported a similar method to ours for the estimation of urinary metadrenaline and normetadrenaline.

Tohoku J. Exper. Med., 1961, 74, 113-129

Climacterium, Climacteric Disturbance and Rejuvenation of Sex Center

By

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INTRODUCTION

No one would object to the proposition that climacterium means the stage of incipient senility, but there are few works giving more concrete attention to it. It is also generally accepted that climacterium begins when the ovary begins to show signs of senescence, but no one has proved it and it is not yet known whether this hypothesis is tenable. The cases of sundry climacteric disturbances are attributed to a "deficiency of ovarian hormones", but we feel justified in raising questions against this generalization. The present authors have been engaged in making researches to solve such questions on climacterium and climacteric disturbances for the past 10 years. In the following, the results of these studies are presented, together with a discussion on the relief from climacterium, that is, rejuvenation of the sex center.

Definition of climacterium and climacteric disturbances

Climacterium is generally defined as the transitional stage between maturity and senility, but such a vague definition is of little help for detailed investigation. Squires[1] gives the definition of "the stage for adaptation to the changes of the ovarian function, accompanying menopause", but in climacterium, not only the ovaries but all the endocrine organs are subject to functional changes. Therefore, the present authors wish to propose the definition of " the stage of abnormal fluctuation of the endocrine functions observed before and after the menopause" for climacterium.

The definition of climacteric disturbances hereunder will be "the diseases mainly consisting of autonomic nervous syndrome observed in climacterium". Accordingly, the climacteric disturbances comprise the symptoms occasioned by disturbance of the autonomic nervous center in the diencephalon due to the dysfunction of the endocrine system and the autonomic nervous syndrome caused by the emotional stress involved in senescence. Hitherto, uterine hemorrhage

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and climacteric melancholy have been included under the climacteric disturbances, but since these may be set aside as climacteric bleeding and involution melancholia, they will be excluded from the following study.

Is the ovary the first organ to show senescence ?

The traditional proposition that the climacteric disturbances are caused by a deficiency of ovarian hormones has misled many authors in the belief that the ovary is the first of all endocrine organs to fall into senescence. When the ovaries of an old white rat above 650 days of age with complete cessation of estrous cycle and that of mature rat were bilaterally exchanged, the sexual cycle of the mature rat was kept intact but the vaginal cycle was not revived in the old rat provided with the ovaries of mature rat (Fig. 1). Therefore, it would seem reasonable to assume that the first organ to fall into senescence cannot be the ovary but some other central hormonal organ. Now, when the hypophysis of a sexually matured female rat is removed, it loses its estrual cycle, but when hypophysis is transplanted from a male rat, it regains the estrous cycle (Fig. 2). It follows that the hypophysis has no sexual difference. As the climacterium is not clearly detectable in male animals, it is difficult to suppose that the climacteric changes begin by occurring in the hypophysis. So, we are finally driven to the hypothalamus containing the sex center as the last resort.

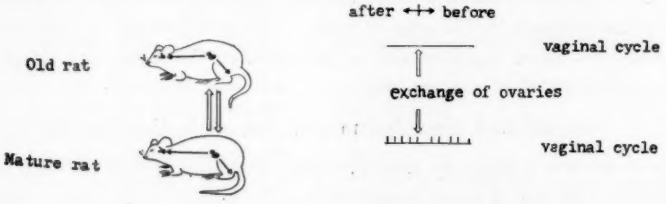


Fig. 1. Vaginal cycle in matured and senile rats after bilateral exchange of ovaries.

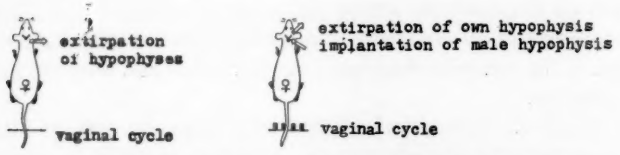


Fig. 2. Vaginal cycle after implantation of the male hypophysis in the hypophysectomized rat.

Senescence of diencephalon

Few, if any, authors have treated the subject of senescence of the diencephalon. The present authors, using the diencephala of old white rats showing no

TABLE I. Secondary Fluorescence of Nucleus Paraventricularis Hypothalamics of the Rat.

	No.	Ganglion cell		Glia cell	Vessel	
		Cytoplasma	Nucleus		Wall	Nucleus
Mature rat	1.	dark orange	dark orange	greenish orange	yellowish green	orange
	2.	〃	〃	〃	〃	〃
	3.	〃	〃	〃	〃	〃
Old rat	4.	brown	brown	greenish brown	greenish brown	yellowish brown
	5.	〃	〃	〃	〃	〃
	6.	〃	〃	〃	〃	〃

more estrous cycles and mature white rats, measured the tissue respiration quotient (QO_2) with a Warburg's manometer, the gomoriphil substance by Halmi's method, the acetylcholine (Ach) content by use of the M. rectus abdominalis of frog and the cholinesterase (ChE) content by Koelle's method, and obtained the results shown in Figs. 3–6.

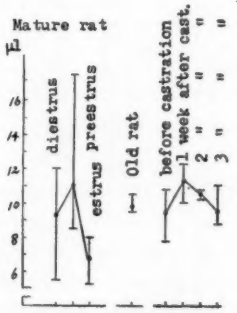


Fig. 3. Qo_2 of diencephalon.

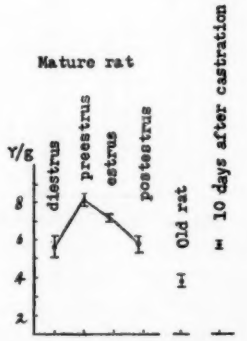


Fig. 4. Ach of diencephalon.

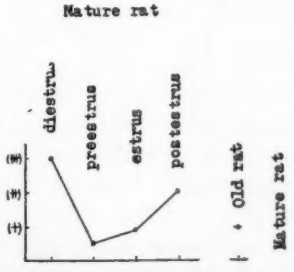


Fig. 5. Gomoriphil substance of hypothalamus.

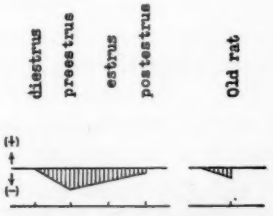


Fig. 6. ChE of nucleus supraopticus hypothalami.

The diencephalic QO_2 was slightly higher in the old rat than in the mature in diestrus, while the Gomoriphil substance and ChE in the old rat were nearly on the same level as the values in the mature rat in estrus, but Ach was lower in the old than in the mature rat in any stage of estrous cycle. The values of QO_2 and Ach were measured in castrated rats and obtained results showed that senescence and castration have different effects on the values. The hypothalamus is influenced by estradiol injection. When mature rats in diestrus were subcutaneously injected with 100 γ of estradiol, the diencephalic QO_2 rose significantly, but in old rats only a slight rise was observed (Fig. 7). In similar tests, the Ach content was considerably higher in mature rats, but rather low in old rats (Fig. 8). When the doses were doubled, the ChE content in the nucleus supraopticus was significantly reduced in mature rats, but only very slightly in old rats (Fig. 9). These results show that when the diencephalon becomes senescent, its sensitivity to estradiol is diminished.

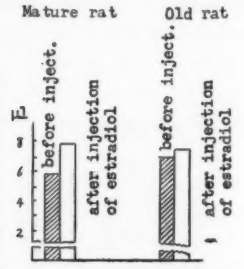


Fig. 7. Qo_2 of diencephalon.

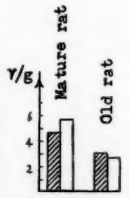


Fig. 8. Ach of diencephalon.

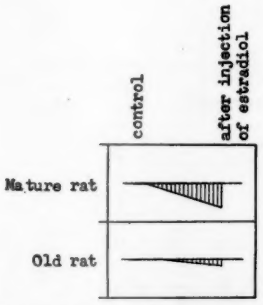


Fig. 9. ChE of nucl. supraopticus.

The secondary fluorescence emitted by young tissues stained with acridin-orange for one minute is greenish, that is the color of rather short wave-length, but in older tissues, the color changes to of longer wave-lengths, through yellow to

orange, brown and finally to red. The diencephalic tissue of old rats, as shown in Fig. 10, showed secondary fluorescences of longer wave-length than those of younger rats. A similar trend was observed in specimens of human diencephalon (Table II and Fig. 2). In the human hypothalamus, the paraventricular, supraoptic and tuberal nuclei, all became senescent at nearly equal paces and the change of the color became particularly evident after menopause, but the fornix showed little change in this respect.

TABLE II. Secondary Fluorescence of Human Diencephalon.

Age	After death	Menstruation	Clinical diagnosis	Ganglion cell of nucl. paraventricularis	Substantia gelatinosa	Fornix
yrs.	hs.					
18	3	(+)	acute leucemia	orange	yellowish green	yellowish green
21	2	(+)	aplastic anemia	〃	〃	green
35	8	(+)	chorionepithelioma	yellowish brown	〃	〃
40	5	(+)	uremia	orange	dark orange	〃
42	2	(+)	sarcoma of larynx	yellowish brown	〃	〃
53	9	(−)	acute leucemia	dark brown	〃	〃
64	2	(−)	subarachnoidal bleeding	〃	〃	〃
64	1	(−)	cancer of esophagus	〃	〃	〃

Note: Acridin-orange staining., 1 min.

Climacteric disturbance and hormones

I) Estrogen: Seeing that climacteric disturbances occur just before and after menopause, castration causing similar disturbances and estrogen effective in curing such disturbances, many investigators believe that climacteric disturbances result from deficiency of estrogen. But upon determination of urinary estrogen of patients with climacteric disturbances, it was found that the value was high in some cases and low in the other showing no definite tendency (Kurzrock[2], Tamis[3], Berger *et al.*[4]). In consideration of the possible periodic rise and fall of the estrogen secretion, the urinary estrogen value of an adult woman and a woman in climacterium were estimated over one month, and it was found that estradiol, estron and estriol were all rather in higher content in the urine of the climacteric woman, as shown in Figs. 12 and 13.

Yamada, one of the co-workers, has made a comparative study of the quantity of estrogen produced upon incubation of the ovary of adult women with gonadotrophin and testosterone, and a similar incubation test with the ovary from climacteric women, and found that the ability of elaborating estogen in most of the climacteric ovaries was not inferior to that of mature ovaries. The

TABLE III. Estrogen Produced after Incubation of 1 g. of Human Ovary with 1,000 γ of Testosterone and Gonadotrophin.

No.	Age	After menopause	Estradiol fraction		Estrone fraction		Total estrogen	
			γ	%	γ	%	γ	%
	yrs.							
1.	46	menstruation (+)	5.0	0.50	5.8	0.58	10.8	1.08
2.	34		7.2	0.72	3.2	0.32	10.4	1.04
3.	39		3.5	0.35	7.0	0.70	10.5	1.05
4.	38		7.4	0.74	5.3	0.53	12.7	1.27
5.	48		7.1	0.71	3.0	0.30	10.1	1.01
6.	43		9.0	0.91	7.0	0.70	16.0	1.61
		yrs.						
7.	44	0.07	13.0	1.30	7.0	0.70	20.0	2.00
8.	48	2	3.7	0.37	0	0	3.7	0.37
9.	56	3	3.0	0.30	3.5	0.35	6.0	0.60
10.	49	0.06	8.1	0.81	7.2	0.72	15.3	1.53
11.	57	2	0	0	0	0	0	0

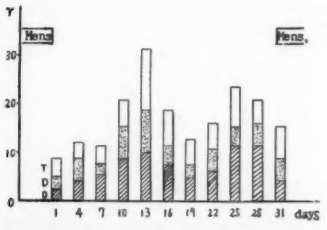


Fig. 12. Urinary estrogens of a normal woman of 32 years.

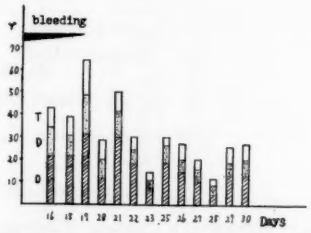


Fig. 13. Urinary estrogens of 46 years old woman with climacteric disturbance.

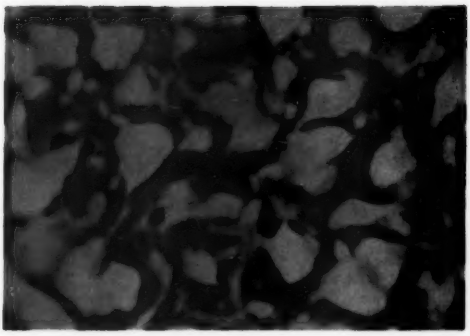
Note: T....estriol D....estradiol O....estron

TABLE IV. Estradiol Produced after Incubation of Rabbit's Suprarenal Gland with 400 γ of Estrone and Gonadotrophin.

	No.	Body weight (g)	Weight of suprarenal gland (mg)	Produced estradiol	
				γ	%
Non-castrated rabbit	1.	2540	315	8.0	2.0
	2.	2010	385	0	0
	3.	1980	235	0	0
Castrated rabbit	4.	2175	336	13.0	3.2
	5.	1875	232	9.0	2.2
	6.	2500	317	14.0	3.0

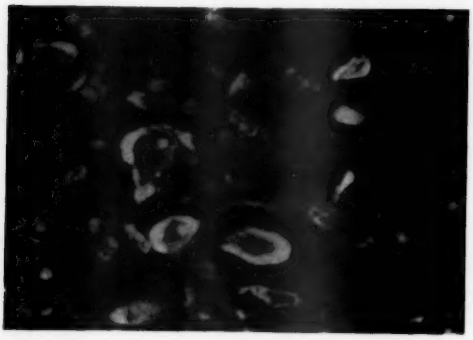
Fig. 10 Secondary fluorescence of rat diencephalon

a) Young rat

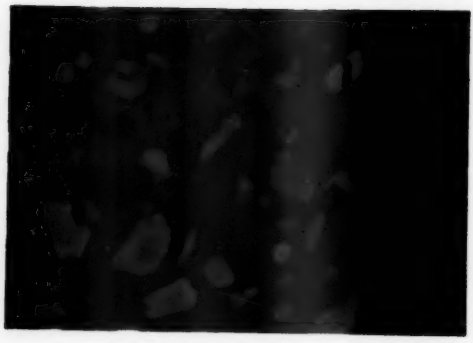


b) Old rat

Fig. 11 Secondary fluorescence of human diencephalon



a) Young woman



b) Old woman

suprarenal gland normally secretes chiefly estrone, but after castration, it secreted estradiol (Table IV). Thus, after oophorectomy, the suprarenal gland replace the ovary in the function of secreting estradiol. These findings show the untenability of the hypothesis that climacteric disturbances are attributed to the effect of decreased estrogen secretion.

II) Gonadotrophin: Numerous authors have reported on the increase of the urinary content of follicle stimulating hormone (FSH) in subjects with climacteric disturbances (Zondek[5], Engel[6], Henderson *et al.*[7], Witschi *et al.*[8], Fluhmann[9], Albright[10], Engelhart *et al*[11], etc.), but the present authors demonstrated that, as shown in Table V the urinary FSH content was not always high in patients with climacteric symptoms. Frank *et al.* state that FSH increases with the appearance of climacteric disturbances and decreases with the disappearance of the syndrome. According to what we have observed, however, as shown in Fig. 14, although some cases showed decrease of FSH with the alleviation of symptoms (A), in some other cases on whom complete remission was obtained by estrogen injection, FSH dropped temporarily with alleviation of the symptoms following estrogen administration but rose again to about twice the pretreatment value on the 14th day when no symptoms were present. From these experimental results and the fact that 80% of climacteric disturbance cases are cured by estrogen therapy while FSH level often increased by rebound phenomenon after estrogen injection, it must be inferred that increase of FSH is not the cause of climacteric disturbances.

TABLE V. FSH in Urine.

Mouse unit per liter	>10	10	20	40	80	160
	Cases					
Climacteric women without disturbance	2	3	2	1		
Climacteric women with disturbance	2	10	10	8	10	4

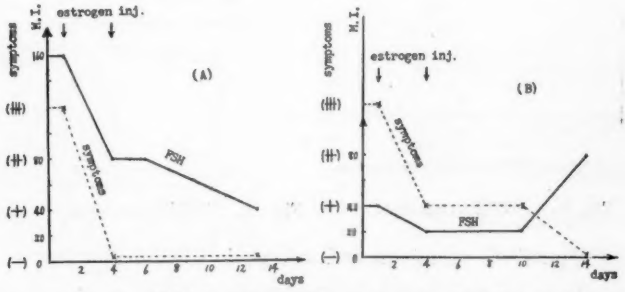


Fig. 14. FSH in urine and symptoms in the climacteric patient subjected to estrogen therapy.

III) Other hormones: No perceptible change was observed in the pregnanediol content in urine of the cases of climacteric disturbances (Fig. 15). The 17-OH-corticoid content in urine was subnormal in some cases but quite normal in others (Fig. 16). The content of 17-KS in urine, ACTH in plasma and TSH-like substance in whole blood were increased only in some of the cases with climacteric disturbances. In short, the hormones in subjects with climacteric disturbances often show abnormal changes, but not enough consistency is found to justify the consideration of these abnormality as the cause of the climacteric disturbances.

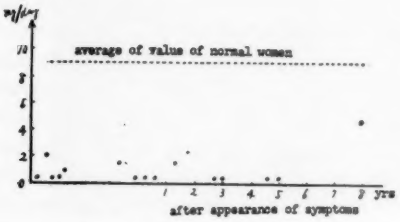


Fig. 15. Pregnanediol in urine of climacteric disturbance.

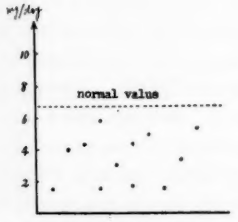


Fig. 16. Glucocorticoid in urine of climacteric disturbance.

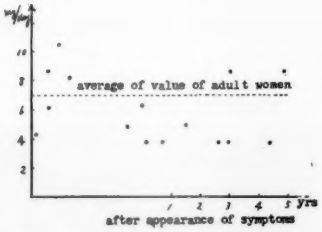


Fig. 17. 17-KS in urine of climacteric disturbance.

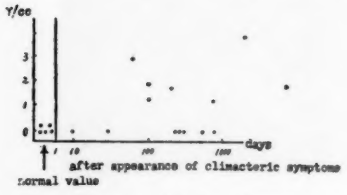


Fig. 18. ACTH in plasma.

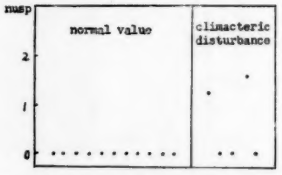


Fig. 19. TSH like substance in blood (10cc)

Climacteric symptoms and autonomic nervous function

As stated above, the climacteric disturbances mainly consist in autonomic nervous syndrome. Accordingly, we proceeded to examine the autonomic nervous

function of women with cilmacteric disturbances. The examination was carried out following the methods which we have devised and which is based on the blood-pressure coefficient (C_B), the pulse coefficient (C_P), calcium test and Motokawa's flicker test.

Let (B) stand for the systolic pressure and (P) for the pulse frequency at recumbent position, and (b) for the systolic pressure and (p) for the pulse frequency at standing position, and C_B and C_P may be determined by the following formulae :

$$C_B = \frac{|B-b|}{B} \qquad C_P = \frac{|P-p|}{P}$$

The mean values of C_B and C_P in normal human subjects were 0.078 and 0.104, respectively. In calcium tests, 0.01 mg/kg of adrenaline was subcutaneously injected and 30 min. afterwards, the decrement of serum Ca was estimated, taking 2.7 mg/dl as the upper limit of the normal value. In the flicker test, the retina was stimulated with electric current of 20 cycles at the room temperature of 18–23°C. The current was gradually increased till flickers began to appear in sight (at S_1 μA) and then it was gradually weakened till the flickers disappeared (at S_2 μA). The flicker value (ΔS) was computed by the following formula :

$$\Delta S = S_1 - S_2$$

TABLE VI. Autonomic Nervous Function of Patients with Climacteric Disorder.

Autonomic nervous function	Normal	Abnormal
C_B (15 cases)	6.7 %	93.3 %
C_P (15)	26.7 %	73.3 %
Ca test (17)	0 %	100 %
ΔS	21.2 %	78.8 %

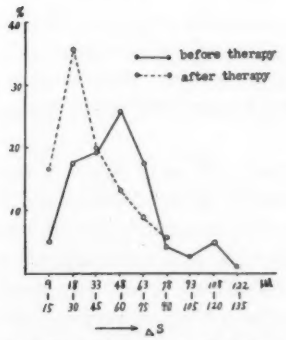


Fig. 20. Changes of ΔS values of climacteric patients.

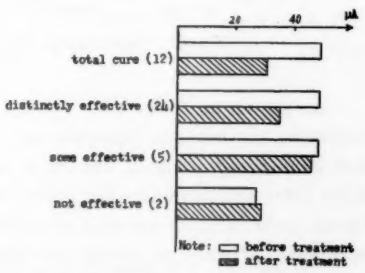


Fig. 21. Efficacy of estrogen and ΔS value.

The normal range of ΔS was taken to be less than 30 μA. The autonomic nervous function, as shown in Table VI, was found to be abnormal in most of the climacteric disturbance cases, though the results of different tests showed no much correlation to each other. The distribution of ΔS values after initiation of therapy for climacteric disturbances, as shown in Fig. 20, shifted to the normal side, that is, ΔS is decreased. Besides this, as shown in Fig. 21, the decrement of ΔS after therapy is increased with the effectiveness of the treatment.

Why is hormone administration effective against climacteric disturbances ?

It has been thus ascertained that climacteric disturbance is less closely correlated with hormone secretion but more with autonomic nervous function, but why is it that hormone administration is effective in treating such disturbances ? As a clue to this question, we tried to evaluate the influence of some hormones on the function of diencephalon, namely, QO_2 and the secretion of Ach, ChE, alkaline phosphatase and Gomoriphil substance. As shown in Fig. 22, the diencephalic QO_2, 6 hours after injection of 1 γ of estradiol, was lowered, rose after 10γ, 100γ and 1,000 γ of it, but the rise after 1,000 γ was not so large as that after 100γ. Progesterone also caused the maximum rise after 100γ. After pregnanediol, known as ineffective as hormone but effective in treating climacteric disturbances, the diencephalic QO_2 increased, but testosterone caused only slight change in the value. When castrated rats were injected with 500 I.U. of serotropin (PMS), the QO_2 value dropped significantly.

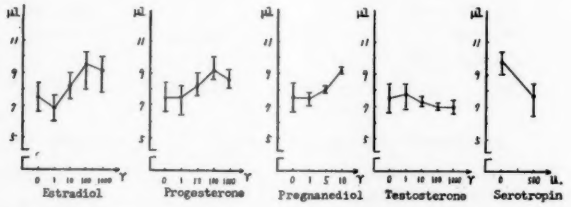


Fig. 22. Diencephalic Qo_2 6 hrs. after hormone injection.

As shown in Table VII, 6 and 9 hours after injection of 0.04γ of estradiol benzoate, the alkaline phosphatase (Al-Ph) content in the diencephalon of rat was moderately lowered, but very perceptibly 9 hours after 0.4γ of estradiol. After 200γ of the latter, the value began to decrease in 6 hours and fell to the lowest level in 12 hours and remained there till the 48 hours, then tended to normalize. Nearly no effect was discernible after 1,000γ of progesterone, and only a moderate decrease of the Al-Ph content was observed 6 hours after 500γ of testosterone propionate.

TABLE VII. Effect of Hormones on Alkaline Phosphatase Content in Nucleus Supraopticus

After injection of hormone		Control	1	6	9	12	24	36	48	72	96 hrs
Estradiol	0.04 γ	0	0	−3	−3	0	0	0	0	0	0
	0.4 γ	0	0	−3	−5	−3	−2				
	200 γ	0	0	−3	−4	−5	−5	−5	−5	−4	−3
Progesterone	1000 γ	0	0	0	−1	0	0				
Testosterone	500 γ	0	−1	−4	−3	0	0				

Note: (−) decrease

As shown in Table VIII, the Gomoriphil substance in the supraoptic nuclei is affected more by the large doses of estradiol than by the small doses of it, but more by the small doses (10γ) than by the large doses (100γ) of testosterone. After 10γ of progesterone, the Gomoriphil substance began to increase in 3 hours, attaining the maximum content in 6 hours, but the content rose abruptly in 12 hours after 1,000γ.

TABLE VIII. Effect of Hormone on the Gomoriphil Substance in the Nucleus Supraopticus

After injection of hormone		Control	3	6	12 hrs
Estradiol	1 γ	+	++	++	+
"	100 γ	+	++	+++	+++
Testosterone	10 γ	+	+	++	++
"	1000 γ	+	+	+	+
Progesterone	10 γ	+	+	++	+
"	1000 γ	+	+	+	+++

Note: (+) increase

As shown in Fig. 23, the diencephalic Ach content was greatly increased after 100γ of estradiol benzoate, but slightly decreased after 1γ- 100γ of progesterone and a little after 5γ of pregnanediol. After 1γ of testosterone propionate, the Ach content fell a little, but sharply increased after 100γ and 1,000γ. In the

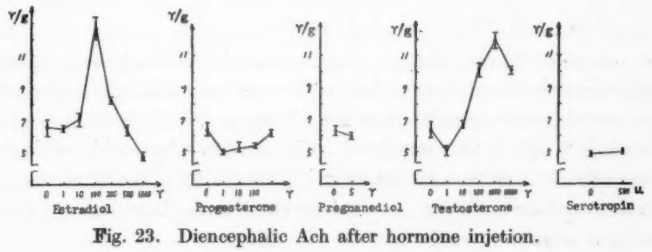


Fig. 23. Diencephalic Ach after hormone injetion.

castrated rat injected with 500 I.U. of serotropin (PMS), the Ach content remained unchanged.

As shown in Table IX, the ChE content in the supraoptic nuclei of the rat was not much affected after 1 γ of estradiol, but increased a little in 3 hours and decreased moderately in 12 hours after giving 10 γ of estradiol. After 100 γ-200 γ, ChE content moderately increased in 3 hours and markedly decreased in 12 hours, the effect being more significant after 200 γ than after 100 γ of the dose. When the dose was raised above 500 γ, the increase at the 3 hours was not observed, while the decrease became apparent after 48 hours. No significant changes were observed after the injection of progesterone or pregnanediol. ChE increased after 1 γ of testosterone propionate and after the doses of 10 γ the increase occurred in earlier stage of observation, although the degree of the increment was rather mild. Injection of 500 I.U. of serotropin (PMS) caused no change in the ChE content in castrated rats.

TABLE IX. Effect of Hormone on Cholinesterase Activity of Nucleus Supraopticus

After injection of hormone		Control	3	6	9	12	23	48 hrs
Estradiol	1 γ	0	0	0	0	−1	0	0
	10 γ	0	+1	0	0	−3	0	0
	100 γ	0	+2	0	−1	−4	−1	0
	200 γ	0	+3	−1	−3	−5	−3	−1
	500 γ	0	+1	0	−1	−1	−2	−3
	1000 γ	0	0	0	0	−1	−2	−3
Progesterone	1 γ	0	0	0	0	+1	+1	0
	10 γ	0	0	0	+1	+1	0	0
	100 γ	0	0	+1	0	+1	+1	+1
	1000 γ	0	0	0	+1	+1	+1	+1
Pregnanediol	5 γ	0		+1	+1			
Testosterone	1 γ	0	+1	+1	+1	+1	+3	+3
	10 γ	0	+1	+1	+2	+2	+2	+1
	100 γ	0	+1	+2	+2	+2	+2	+1
	1000 γ	0	+1	+2	+2	+2	+1	0
Serotropin	500 I.U.	0		−1	0			

Note: (+) increase (−) decrease

Thus, it was found that the factors governing the diencephalic function—QO_2, Al-Ph, the Gomoriphil substance, Ach, ChE — are all under the influence of the hormones tested, either positively or negatively, and in particular, the contents of Ach and ChE, which are considered to have close relationship with the function of autonomic nerves, are strongly influenced by the hormones. Besides this, the more effective hormones against climacteric disturbances showed the more stronger effect on the diencephalic function.

Is rejuvenation of the sex center possible ?

Although the sex center is now generally considered to be located in hypothalamus, it is not decided which nuclei represent the center as yet. In this study, therefore, the part of hypothalamus containing the supraoptic, paraventricular and tuberal nuclei was taken as to represent the sex center.

As stated above, when the hypothalamus becomes aged, the secondary fluorescence after acridin-orange staining will have longer wave-length. Hence, we adopted the method of comparing the secondary fluorescence from so-called sex center of old rats of same maturity, devided into experimental and control groups, for the evaluation of rejuvenative effects of various agents. By such a method, it was demonstrated that royal jelly and orotic acid had the rejuvenative effect.

DISCUSSION

There is no disagreement to the fact that climacterium is the commencement of senescence, however differences of the opinion exsist in regarding the generally accepted hypothesis of the senescence initiating in the ovary. Our investigators led to the conclusion that senescence begins rather in the hypothalamus, and that this process can be alleviated by the administration of royal jelly or orotic acid. Sex center is located in the hypothalamus, hence it could be deduced that these two agents also exerts their rejuvenating effect on the sex center. When royal jelly is injected to women in menopausal stage, menses-like hemorrhage is sometimes observed.

Hitherto, it has been generally accepted that climacteric disturbances are caused by deficiency of estrogen due to retrogression of the ovarian function. But the excretion of estrogen in urine was found not always decreased in patients with climacteric disturbances. In these cases, the follicle-stimulating hormone (FSH) was found to be increased in many cases but in some cases this increase was not found. Besides this, there are cases in which moderate increase of the hormone was found but without any symptom of climacteric disturbance. Thus, the change of FSH content did not show parallelism with the manifestation of such symptoms, neither their contents of 17-OH-corticoid, 17-KS, ACTH and TSH showed any correlation with the grade of climacteric disturbances. Yet, almost all the patients showed instability of the autonomic nervous function. From these findings, we may infer that climacteric disturbances are caused by a primary instability of the autonomic nervous function brought about by the aging of the nervous center and a secondary dysfunction of the autonomic nervous center due to the senescence of the hormonal organs. It is hardly possible to maintain the endocrine balance permanently on the level of full maturity, but even without bothering about the hormonal balance, we may correct the dysfunc-

tion of the autonomic nervous center. For treating climacteric disturbances, repair of the impaired function of the autonomic nervous center should be the objective. The effectiveness for repairing the autonomic nervous function is the strongest in estrogen, next in testosterone, weak in progesterone and barely detectable in pregnanediol. Gonadotrophin also proved effective, but only slightly. The efficacy of these hormones in repairing the impaired autonomic nervous function runs nearly parallel with their efficacy against climacteric disturbances. Slow intravenous injection of 0.5% solution of procain chloride, known as devoid of hormonal effect but effective in regulating the autonomic nervous function, also proved as effective as estrogen injection in alleviating such disturbances.

The current policy of treating climacteric disturbances by attempting replenishment of deficient hormones is misdirected. When some hormonal treatment is contemplated then, the aim of the treatment must be the autonomic nervous center. Consequently, hormone treatment against climacteric disturbances need not be continued long, a course of hormone administration of less than a week being often sufficient for the purpose. If such a short course proved to be ineffective, some other therapeutic procedures should be resorted to. When 3–4 different methods in succession prove to be utterly unsuccessful, then the case undoubtedly represents a case of disturbances caused by emotional conflict, and is uncurable unless she is duly treated as a psychosomatic patient. The subject of psychosomatic disorder will be treated in another report.

CONCLUSION

The present authors' investigation on climacterium, climacteric disturbances and the possibility of rejuvenating the sex centers led to the following conclusions :

1. The hypothalamus falls into senescence earlier than the ovary. Therefore, the currently accepted opinion that climacteric changes begin in the ovary and then proceed to the hypothalamo-hypophyseal system is unfounded.

2. Some of the climacteric disturbances are psychosomatic and some are endocrino-vegetative. The results of our study led us to conclude that the latter are not necessarily correlated with deficiency of estrogen or increases of gonadotrophin, but from a dysfunctional syndrome of the autonomic nervous center due to the effect of senescence of the diencephalon and the hormonal organs.

3. Estrogen proved to be most effective in correcting such a dysfunction of the autonomic nervous center, but androgen, gonadotrophin, pregnanediol, and even the nonhormonal procaine are also effective.

4. The objective of treating climacteric disturbances is to correct the dysfunctioned autonomic nervous center. It is impossible to expect success after long persistent treatment with hormones only. When a course proves unavailing within a week, another therapy must be contemplated, and if 3–4 methods fail

to effect, psychosomatic disorder should be suspected. The doses of the hormones used need not be large, 1 mg of estrogen, 10 mg of androgen or 10 mg of procaine per day will be quite sufficient for the purpose.

5. It was demonstrated that treatment of climacteric disturbances should succeed by merely bringing the autonomic nervous function back to order, without attending to the function of the sex center, and also that the attempt at rejuvenating the hypothalamus, the seat of the center, is a hopeful enterprise.

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Psychosomatic Diseases in Woman

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INTRODUCTION

Some patients who suffer from climacteric disorders do not respond to the treatment currently available. Detailed examination of these cases revealed that a large part of these patients had emotional frustrations. This finding led authors to investigate the psychosomatic disease (PSD).

Although numerous discussions on PSD were presented since the reports of Dunbar[1] and English & Weiss[2], no clear cut definition of PSD has been proposed. Some investigators in the field, furthermore, confuse the issue with the use of the term of PSD and psychosomatic medicine (PSM) interchangeably. The authors of this report propose the definition of PSD and PSM as follows.

Human diseases are caused not only by the somatic causations but also the psychic factors. Moreover, patients who suffer somatic disease will receive significant influence by the disease upon their psyche which, in turn, reflects it's changes, thus induced, upon the patient's soma. Thus, all the diseases and patients should be understood not only from the view point of somatic changes but psychic aspect as well. Regrettably, present days medicine still lacks this attitude and the medical science which stress this point should be designated as PSM. The other hand, PSD could be designated as "somatic symptoms induced by the emotional frustration". Namely PSM takes all diseases and somatic changes as it's objects and PSD concerns with only limited cases who manifest somatic symptoms caused by emotional factors, Neurosis, the other hand, is also caused by emotional frustration but in this instance, psychopathic tendency plays a dominant role.

PSD in women

Among 13170 patients who visited our gynecological and obstetrical clinic, PSD was found in 194 namely in 1.5%. The list of clinical diagnosis is presented in Table I.

Symptoms identical with the climacteric disorders appear not only in the climacterium but also in any stage of woman's life, including puberty, premenstrual stage, menstruation, pregnancy, puerperium, postabortion and in old

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Table I. PSD in our Clinic

Clinical diagnosis	No. of cases	Clinical diagnosis	No. of cases
Oligomenorrhoea	1	Pelvic congestion	8
Hypermenorrhoea	2	Parametropathia spastica	9
Dysmenorrhoea	5	Climacteric disorder-like symptoms	91
Irregular uterine bleeding	4	Enuresis nocturna	4
Lumbago and abdominal pains	8	Irritable bladder	8
Pruritus vulvae	5	Corpulence	2
Leucorrhoea	5	Excessive vomitting	12
Vaginism	1	Abortion	3
Coital pains	4	Emotional hypertension	1
Dyspareunia	13	Imaginary pregnancy	1
Excessive libido	2		

age. The present authors termed these symptoms as "vegetosis". Most frequently encountered type in PSD was psychosomatic vegetosis which occupied 44.8% of all PSD. The authors were able to find definite psychological factors in all of these patients and this analysis revealed that the most frequent cause to be the trouble with husband which occupied 25% of all cases. Sexual immorality complex caused PSD in 19%. Family conflict which occupied the 4th rank and 12% may be contribute to the unique circumstances of Japanese family system (Fig. 1).

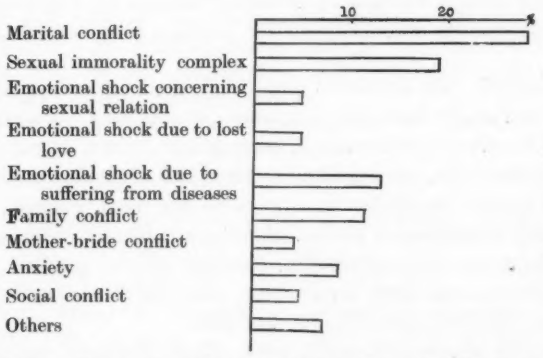

Fig. 1. Causes of emotional frustration in female PSD Cases.

Diagnosis of PSD

As the above definition of PSD indicates, we must find out what is causing the emotinal frustration in the patient in diagnosing PSD. At our clinic, the following procedure is adopted to meet this requirement:

1) Clinical examination: Every tests related with the patient's complaints

is carried out without omission. In PSD, such tests never reveal any change that may explain the subject's complaint, and suspicion of PSD should be raised at once. In general, the symptoms complained of in PSD are variegated, and the behavior of the patient in describing them is always full and circumstantial, as if trying to force the physician to accept her assertion that she is in sober fact suffering from some disease. And yet no somatic change affording ground for such complaints can be elicited. Drugs and physical treatments are utterly ineffective against such symptoms. Such cases can be diagnosed as PSD with rare exception. But an interview should not be neglected before telling a sure diagnosis.

2) Interview: A PSD patient always suffers from a deviation of personality. To detect this, her life history has to be studied. The interview should cover the details of the health of her mother during her fetal life, whether she is the first born or not, her childhood reminiscences, her relation to parents and brothers and sisters, her school life, her feeling of menarche, her occupation and environment at the job site, fate of her love affair, if any, if married, how she felt at her first nuptial bed, her stand in sexual life, her relation to the family members, economical stand, religion, past diseases, habits and peculiarities, taste and hobbies, how she evaluates her own personality and what are her frequently dreamt dreams etc., to investigate whether she had dissatisfaction, displeasure, surprise, troubles, disappointment or any such mental shocks concerning these items. When patient is hesitant in discussing matters on which she has strong repression, such matters concerning sex for instance, then narcoanalysis may be resorted to.

In narcoanalysis, the subject is questioned after slow intravenous injection of penthotal sodium to losen the repression, on matters concerning sex and matters hard to educe in interview in normal state. Such an examination will usually reveal more than one emotional stresses, but when the mention of any of these signally touches the subject so as to cause her weep, mumble, look sad, or become suddenly loquacious or excited, this stress is taken to represent the cause of her somatic signs. The information obtained on the question whether the somatic symptoms came forth immediately after the frustration or some time thereafter also offers help for unerring diagnosis.

3) Shagass's sedation threshold test: Many diagnostic methods for PSD have been proposed but few are proved to be useful. But we found that the sedation threshold test reported by Shagass[3] in 1954 is helpful in diagnosing PSD. The electroencephalogram (EEG) induced from the bilateral lobi frontales, begin to show quick waves of 15–30 cycles/sec. and describe spindle waves when amobarbital is injected slowly into the vein. During the injection at the rate of 0.5 mg/kg/40 sec. spindle waves soon begin to appear. With the increase of the injected dosis, the mean amplitude of the waves becomes larger, but when

the dosis reaches a certain level, the rise of amplitude stops. This level represents the sedation threshold (ST). The results of the tests for the values of ST in controls, cases of PSD diagnosed by psychoanalysis and successfully treated PSD cases are shown in Tabs. II, III and Fig. 2. We see that in the controls, the ST was 3.5 mg/kg at most; in PSD cases, however, it rose to 7 mg/kg at the highest, and even in the lowest case, stood at 4 mg/kg. After treatment, when PSD was successfully treated, the ST fell to 3 mg/kg, but when the treatment was of no avail, the value remained at 6 mg/kg. Such results show the effectiveness of ST determination in making diagnosis of PSD.

Table II. Sedation Threshold (ST)

Control

Name	Age	Diagnosis	ST (mg/kg)
K. E.	25yrs.	Habitual abortion	3.5
K. T.	46	Lumbago	3
T. T.	24	Vegetosis	2
S. M.	36	Parametropathia spastica	2

PSD

Name	Age	Frustration	ST (mg/kg)
H. T.	35yrs.	Organ affected conflict	7
S. M.	32	Cancer phobia	6
T. A.	35	Anxiety for sickness of baby	6
A. E.	31	Conflict with husband	6
M. N.	31	Anxiety for sterility	5
T. K.	54	Conflict with son	5
D. Y.	20	Organ affected conflict	5
K. T.	48	Occupational conflict	4
Y. S.	29	Mother-bride conflict	4

Table III. ST before and after Hunger Therapy of PSD

Name	Age	ST before therapy	ST after therapy	Effect of therapy
I. T.	21yrs.	8 mg/kg	3 mg/kg	healed
H. F.	42	8	6	no effect
H. M.	46	5	3	improved
K. A.	40	5	2	healed
N. T.	45	4	3	healed

ST appears just when the patient falls into the slurred speech, so that in clinical practice, the patient may be asked to count backward from 100 while receiving intravenous injection of amobarbital, and its dosis (in mg/kg) given to the moment when the counting becomes confused may be taken as the ST.

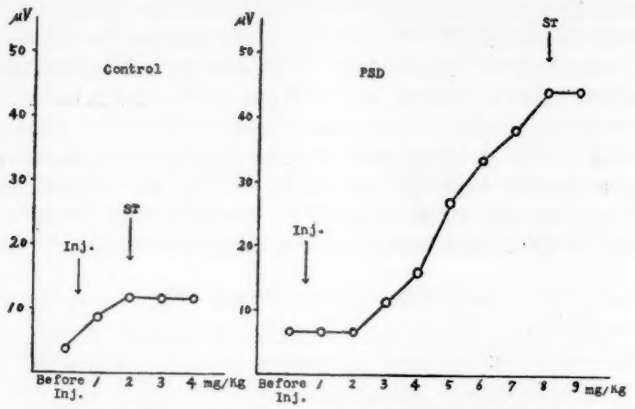

Fig. 2. Electroencephalographic amplitude of the frontal lobe in microvolts.

Treatment of PSD

It has been accepted that the insight therapy which aims at making the patient patently conscious of the latent conflict disclosed by psychoanalysis is most effective for treating PSD, but this therapy is not effective when the intelligence of the patient is low or when it is more advantageous for the life of the patient to remain in PSD. Besides, success in psychoanalysis is assured only in the presence of an experienced psychotherapist. For this reasons, continual sleep therapy or electroshock therapy etc. have been often practiced, but these are of low efficacy after all. The present authors have obtained successful results by means of our new hunger therapy described below.

The previously advocated hunger therapy is a very effective method of treatment, but hunger causes so much distress that not all patients can stand it for the prescribed period. We administered chlorpromazine to assuage the sting of hunger as follows: 12.5 mg of it was injected intramuscularly twice daily on the 2nd to 6th days of fasting; when the discomfort is not sufficiently suppresed, the dosis is doubled to 25 mg each. After the 7th day, 12.5 mg is given intramuscularly only when discomfort in the abdomen is complained of. During hunger therapy, stool occult blood test became positive at around the 7th day of treatment. Occasional complications such as gingival bleeding and angular stomatitis may occur. To prevent these complications 60 mg of methylhesperidin, 60 mg of rutin, 150 mg of vitamine C, 3 mg of vitamine K, 3 mg of adrenochrome monosemicarbazone and 10 mg each of vitamine B_1 and B_2 are given daily. Many cases complain of abdominal swelling after the 7th day. This could be countered with the daily per oral administration of 0.03 g of menthol. If the abdominal

swelling persists, soap water enema (500 cc) will help. Through the period of fasting, about 3000 cc of water is given; in the recovery period, the water intake is reduced to about 2000 cc. This is schematically shown in Fig. 3.

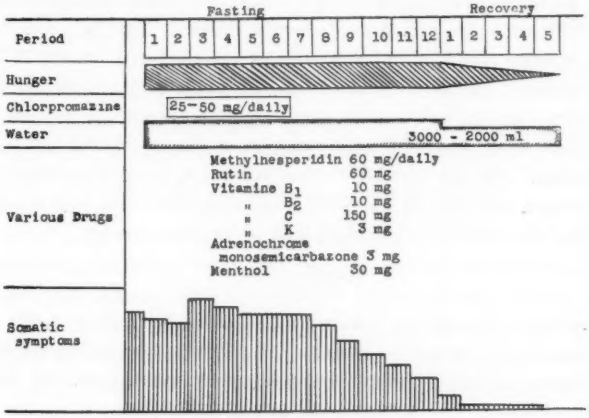

Fig. 3. Gist of hunger therapy.

The menu in the recovery stage, as shown in Tab. IV, is made to increase caloric intake gradually. Sudden ample feeding must be avoided, for it may cause shock and death of the patient. The fasting is ordinarily kept up for 12–15 days and 5 days are usually given for recovery.

In the recovery stage of the fasting, the patient becomes very obedient to the physician. If, therefore, psychotherapeutic measures such as persuasion and encouragement etc. were taken in this stage, then it will effect more favorably

Table IV. Diet Schedule of Recovery Stage

Date	Breckfast	Lunch	Supper
1		A cup of milk	A cup of milk
2		A dish of gruel	A dish of gruel
3	Fasting	Milk and gruel	Milk and gruel
4		Biscuit and milk	Biscuit and milk
5		A half dish of usual fare	A half dish of usual fare
6	Usual fare	Usual fare	Usual fare

Table V. Therapy of PSD

Therapy	No. of cases	Healed	Improved	No effect
Hunger therapy	57	23 (40.4 %)	27 (47.4 %)	7 (12.3 %)
Psychotherapy	130	13 (10.0 %)	78 (60.0 %)	39 (30.0 %)

on the patients.

This new therapeutic method had been applied to 57 PSD cases and resulted in perfect healing in 23 cases and good effect in 27 cases, or commendable efficacy in 50 cases in total (87.7%). Evaluation after several months revealed that 40 had completely recovered and 6 were greatly relieved, that is, good results were reported in 46 cases (70.2%), but relapse was reported in 11 cases (19.3%). The majority of cases of relapse were those of PSD caused by environmental factors, and their return to the unimproved environments apparently caused the recurrence. In such cases, therefore, improvement of the environmental factors must be also attended. Of the 130 PSD cases subjected to only psychotherapy, the treatment proved effective in 91 (70%) and complete cure was reported in 13 (10%), so that the efficacy is less than that of hunger therapy.

CONCLUSION

According to the author's definition of PSD as "the somatic symptoms induced by emotional frustration as cause", the PSD cases visiting the Gynecological and Obstetrical Clinic, Tohoku University, were investigated and the following results were obtained.

1. In 13170 patients PSD was found in 194 cases (1.5%).

2. The most frequent type of PSD was vegetosis, i.e., climacteric disturbance-like disease, occupying 44.8% of total PSD.

3. The most frequent type of mental frustration was the unhappy marital conflict (25%), followed by the sexual immorality complex. Conflict with other family members also occupied the fourth place in the list, and frustration due to unhappy mother-bride relation may be attributable to a specific feature of the family life in Japan.

4. PSD can be clinically diagnosed by the absence of any actual change responsible for the somatic change complained of, the insistence of the patients in sustaining her complaints, the utterly irrational complaints, the ineffectiveness of any medicaments and physical therapy against the complained symptoms and so forth, however, the existence of PSD could be made with the detection of mental frustration by narcoanalysis. Shagass's sedation threshold test is also helpful in diagnosing PSD.

5. For treatment of PSD, combined use of our new hunger method and elementary psychotherapeutic procedures proved most effective.

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Prolonged Slow Negative Wave in Primary Sensory Evoked Potentials of Dog Cerebral Cortex

By

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Since Iwama and Jasper[1] reported the action of γ-aminobutyric acid (GABA) upon the primary sensory evoked potential of the cerebral cortex, many works have been done on this subject. It is now generally known that only the 10–30 msec surface-negative wave of the evoked potential is diminished by topical application of GABA, leaving the initial positive wave intact or augmented. However, when a detailed observation is made on the evoked potential under the action of GABA in non-anesthetized animals, it is very often found that a prolonged surface-negative wave 100-150 msec in duration appears following the initial surface-positive wave.

Since no extensive studies on the slow negative wave of the evoked potential under the influence of GABA have been available, it was attempted to disclose properties of this sort of the evoked activity. In the course of the study, however, it was found that a similar prolonged negative wave could be produced as a component of the sensory evoked potential without application of GABA in non-anesthetized animals.

While this study was being carried out, the paper by Pearlman and others[2] dealing with the slow negative wave in the visually evoked cortical potentials called our attention. The slow negative wave studied by them seems to be essentially identical with that reported in this paper. Though the experimental findings obtained in the present study are in good agreement with those of the previous workers, it seems still worth while publishing this paper, because many points which remained unattacked have been elucidated in this study.

METHOD

All experiments were performed on adult dogs. Ether anesthesia was used during early stages of the operations, and then the animals were kept immobilized by d-tubocurarine chloride (0.2–0.3 mg/kg/hr. intravenously) and maintained on

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artificial respiration. Evoked potentials of the cerebral cortex were usually obtained by stimulating the thalamic somatosensory relay nuclei (n. ventralis posterolateralis and n. ventralis posteromedialis) with a concentric bipolar electrode 0.5 mm in diameter, which was inserted stereotaxically consulting the atlas of the dog's brain.[3] Stimuli were rectangular pulses of brief duration less than 10 volts in strength. Recording electrodes were a silver wire with a ball tip of 1 mm diameter for the cortical surface and a glass pipette (10 μ in tip diameter) filled with physiological saline for the deeper layers of the cortex. The reference electrode was a screw fixed to the skull over the frontal sinus. The electrical activities were amplified with condenser-coupled amplifiers (time constant : 1.4 sec) and led to a dual beam oscilloscope. Ink-writing tracings were simultaneously obtained.

The chemical agents used for modifying the evoked cortical potentials were 1% GABA, 1% KCl and 0.1% strychnine in physiological saline, all of which were applied topically to the exposed cortical surface.

RESULTS

It is well known that the primary evoked potential to a single shock in the sensory nucleus of the thalamus or peripheral sensory nerves consists of an initial surface-positive wave (P-wave) followed immediately by a surface-negative one (N-wave). In curarized, non-anesthetized dogs this diphasic primary complex was followed by a prolonged surface-negative slow wave (SN-wave). Its duration was as long as 150 msec or more (Fig. 1, A; bottom record) and the amplitude measured about 500 μV in the best condition. From some points of the sensorimotor cortex only the SN-wave was observed without being preceded by marked P- and/or N-waves (Fig. 1, B). The most excitable points for the SN-wave did not necessarily coincide with those for the primary complex. This suggests that the development of the SN-wave is not directly related to that of the primary complex. However, the cortical areas giving rise to the SN-wave in response to thalamic sensory stimulation were confined almost exclusively to the somatic afferent area which has been defined classically.

I. Amplitude of the SN-wave as functions of intensity and frequency of stimulation

As illustrated in Fig. 1, among the primary complex and the SN-wave, the latter appeared first when the stimulus intensity was gradually increased from the sub-threshold level. Throughout the present study it was unfailingly found that the threshold for the SN-wave was slightly lower than that for the primary complex. The stimulus voltage for the maximal SN-wave was also lower than for the maximal primary complex. These observations are in complete agreement

with the finding by Pearlman *et al.* As the primary response attained its maximal amplitude, the SN-wave was definitely diminished (Fig. 1, A). This may

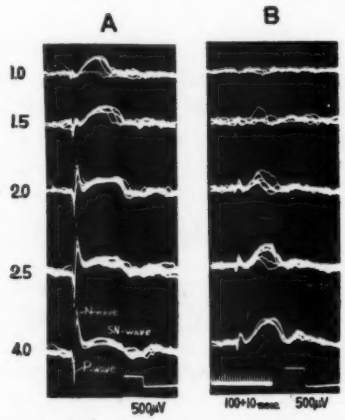

Fig. 1. Series of evoked cortical potentials to a single shock applied to thalamic VPL, showing effects of increasing stimulus intensity. Stimulus intensities are given to the right in arbitrary scale. All records were formed by superposition of 5 to 7 traces. A: Responses recorded from a most active site. Note, only SN-wave appeared to low voltage stimulation and as primary complex developed some masking effect on SN-wave obtained. B: Records obtained simultaneously with A from a locus a few mm apart from the point for A. Development of primary complex was less marked and its masking effect upon SN-wave was very little. Negativity upward in this and all subsequent figures.

be interpreted to mean that there is some masking effect of the primary complex upon the SN-wave, reducing the amplitude of the latter. This supposition is supported by the records in column B in Fig. 1 which were taken at a locus a few millimeters apart from the site most active for the primary complex. In this case the development of the primary complex is not marked, so that its masking effect upon the SN-wave was very little, resulting in a continuous increase of the SN-wave with increasing the stimulus intensity.

When the thalamic sensory nucleus was repetitively stimulated, the amplitude of the SN-wave was notably affected. In Fig. 2 a representative instance is shown. In this experiment a series of stimuli with intensity just maximal for the SN-wave were applied to the thalamic VPL at varying frequencies. At a low frequency less than 0.5/sec, the evoked SN-waves were considerably variable in amplitude (Fig. 2, A). By using a little higher frequency (0.7–2/sec), however, the variability of the SN-wave was reduced and its amplitude was augmented (Fig. 2, B). When the stimulus frequency was further increased up to 3/sec,

the SN-wave responded in a waxing and waning manner (Fig. 2, C). The period of the waxing and waning of the amplitude was variable from case to case and

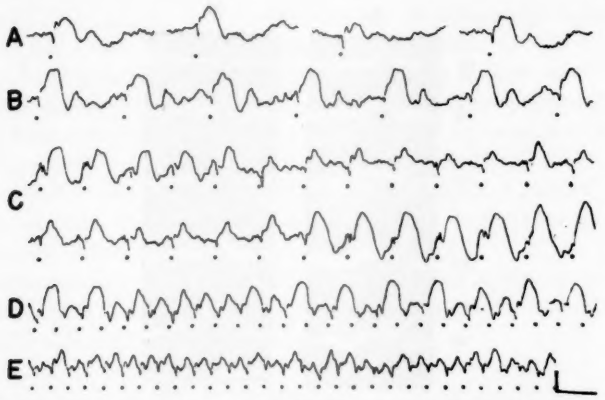

Fig. 2. Responses evoked by repetitive stimulation of VPL at 0.3/sec (A), 1.5 /sec (B), 3.7/sec(C), 6.3/sec(D) and 8.5/sec(E). SN-waves were variable in A and were stabilized and slightly augmented in B. In C, SN-waves appeared in a waxing and waning manner. Upper and lower records are continuous. In D, high and low amplitude responses appeared alternately. With further high frequency (E) SN-waves were almost completely suppressed. Calibrations: 400 μV and 250 msec.

also from trial to trial in one experiment. This response pattern to repetitive stimulation could be observed at a frequency as high as 7/sec. In addition, it was observed with this stimulus frequency that the SN-wave appeared with high and low amplitude alternately at the waxing stage (Fig. 2, D). Using a further high frequency, the SN-wave was almost completely suppressed, while the primary complex was still recognizable (Fig. 2, E).

II. Effects on the SN-wave of barbiturate anesthesia and of reticular formation stimulation

When the animals were left immobilized for several hours, it was a usual observation that EEG was altered from low voltage fast waves to high voltage slow waves. In association of such spontaneous change in EEG activity the amplitude of the SN-wave became very variable (Fig. 3, a), presumably because of the spontaneous slow waves prevailing in the background. Though such was the case with the SN-waves, the primary complex remained comparatively stable or in some cases it was augmented. A similar observation could be made by administrating a small dose of barbiturate to curarized animals in full alertness. Usually an intravenous injection of 10 to 20 mg/kg barbiturate was sufficient to induce the spontaneous slow waves in EEG, making the evoked SN-wave unstable.

A further addition of barbiturate up to 50 mg/kg in total dose was found to suppress the SN-wave almost completely. Such a high dose of barbiturate suppressed the N-wave considerably, and the P-wave was also affected though to a lesser extent.

At the stage in which the SN-wave became unstable spontaneously or by injection of a small dose of barbiturate the effects of high frequency stimulation of the mesencephalic reticular formation (RF) upon the SN-wave were examined. During stimulation of the RF the P-wave was always suppressed and EEG altered

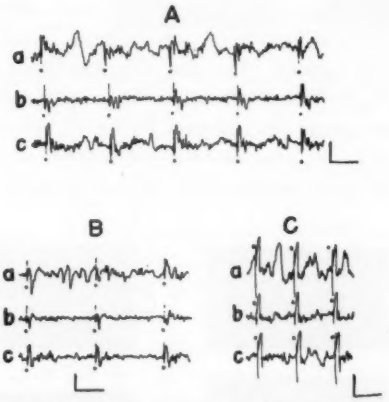

Fig. 3. Effects of RF-stimulation. Frequency of stimulation, 200/sec. Pulse width, 0.05–0.3 msec. Intensity, 5 volts. In each record, a : before, b : during and c : immediately after the stimulation. Sharp upward deflections indicated by dots are N-waves. Calibrations : 400 μV and 1 sec.

to show the arousal pattern. The effects on the N- and the SN-waves, however, varied from one case to another. These effects could be classified into three types : 1) Both the N- and the SN-waves were suppressed (sample record not illustrated). Usually the effect on the latter was less marked than that on the former. 2) The SN-wave was suppressed with the N-wave unaffected. In some cases the SN-wave was gradually released from suppression during the stimulation (Fig. 3, A), and in others the suppression persisted as long as the stimulation continued (Fig. 3, B). 3) The N-wave was remarkably suppressed, but the suppression of the SN-wave was very slight (Fig. 3, C). Taking these data altogether, it seems that the SN-wave behaves under the reticular activating influence independently of the N-wave.

When reticular stimulation was stopped, the potential waves, if suppressed during the stimulation, were recovered rapidly to the pre-stimulation level and were kept stabilized during the sustained EEG-arousal. In some cases a transient

augmentation (rebound) of the evoked potential was observed in all the components immediately after the stimulation was withdrawn.

III. Modifications of the SN-wave by asphyxia and by topical application of KCl, strychnine and GABA

Asphyxia: The susceptibility of the SN-wave to asphyxia was studied in comparison with that of the primary complex. With an animal immobilized with d-tubocurarine artificial respiration was stopped. Two to three minutes later the N-wave was almost completely abolished, while the P- and the SN-waves were still present though their amplitudes were reduced to about one half of the control and both were a little prolonged in duration (Fig. 4, B; upper trace). Three to four minutes after the commencement of asphyxia the SN-wave disappeared (Fig. 4, B; lower trace). Then, artificial respiration was again started. Within one minute the SN-wave recovered almost to the control level (Fig. 4, C). Five to seven minutes after resumption of ventilation, the entire potential complex returned completely to the control level (Fig. 4, D).

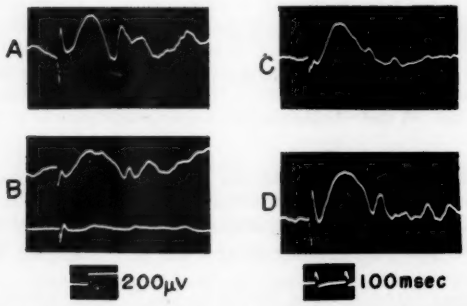

Fig. 4 Effects of asphyxia. A: Control response. B: Upper and lower traces are responses 3′ and 4′ after stopping artificial respiration, respectively. C: 1′ after resumption of respiration. SN-wave recovered more rapidly than primary complex. D: Full recovery of all components of evoked potential about 4′ after record C.

KCl: Suppression of the SN-wave, similar to that seen in asphyxia, was obtained by topical application of 1% KCl solution. Fig. 5, B_1 is a record obtained two and a half minutes after KCl application. By this time the N-wave had completely been abolished, while both the P- and the SN-waves remained almost unaffected. About five minutes later, the SN-wave was virtually abolished (Fig. 5, B_2). After record B_2 was obtained, the cortical surface was rinsed with warm saline solution. Four minutes later, the SN-wave recovered considerably though the primary complex was still suppressed (Fig. 5, C_1).

Within ten minutes the SN-wave completed a full recovery, but the primary complex remained reduced in size (Fig. 5, C_2).

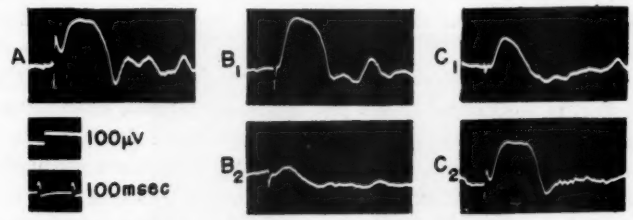

Fig. 5. Effects of topical KCl. A : Control response. B_1 and B_2 : 2′30″ and 5′45″ after KCl treatment. N-wave was suppressed earlier than SN-wave. C_1 and C_2 : Recovery at 3′42″ and 10′, respectively, after washing off the brain. SN-wave recovered earlier than primary complex.

Strychnine : A solution of strychnine 0.1% in concentration was applied topically at a cortical point where the primary complex and the SN-wave developed moderately (Fig. 6, A). With a lapse of time the primary complex, especially its N-wave, was gradually augmented (Fig. 6, B), and finally it turned into a triphasic (positive-negative-positive) potential wave of very high amplitude (Fig. 6, C). The evoked potential so modified by local strychninization was very similar in wave form to the spontaneous strychnine spike which began to appear at the time of record C of Fig. 6. In contrast to this, the SN-wave could be traced on the declining phase of the augmented negative wave even at the final stage of strychninization. It seemed that the SN-wave did not constitute a main component of the evoked potential augmented under the influence of strychnine.

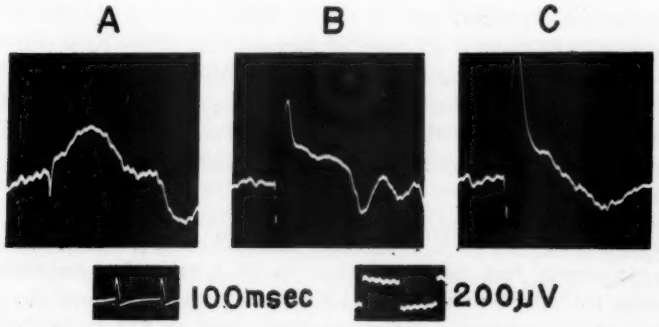

Fig. 6. Effects of topical strychnine. A : Control response. B and C : 5′30″ and 11′ after local strychninization, respectively. At the time of record C, spontaneous strychnine spikes were observed. In C, SN-wave appeared as a notch on descending limb of augmented negative wave.

GABA : The effects of topical application of GABA were examined in non-anesthetized animals showing the primary evoked potential with a good SN-wave. An example of the results is shown in Fig. 7. In records A and B the upper and the lower traces show the responses from the surface and the deep layer 1 mm beneath the surface, respectively. The control response is shown in record A and the response obtained 15 sec after topical application of 1% GABA in record B. In conformity of the previous works, GABA promptly abolished the N-wave and increased the P-wave in amplitude and duration. In addition to this, it was observed that GABA augmented the SN-wave extremely. Corresponding to the modification of the surface response, the slow wave of positive polarity, which was found by the deep recording to be associated with the surface SN-wave, was significantly increased in amplitude by GABA.

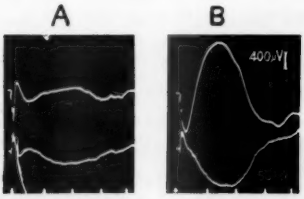

Fig. 7. Effects of topical GABA. In each set, upper and lower traces show surface response and response led from 1.0 mm beneath the surface, respectively. In B, SN-wave was extremely enhanced by surface application of GABA. Note simultaneous augmentation of deep slow positivity.

Augmentation of the SN-wave due to topical application of GABA, may be attributable partly to suppression of the N-wave which, as stated in the previous section, masks a part of the SN-wave. If only the removal of the masking on the SN-wave is the cause of augmentation of the SN-wave, it is expected that the SN-wave may also be enhanced, though transiently, when the N-wave was progressively eliminated by KCl applied on the cortical surface. But such was not the case. On these bases it may be concluded that in addition to the removal of the masking, there must be an augmenting action of GABA upon the SN-wave.

IV. Intracortical distribution of the SN-wave

It has already been mentioned that the SN-wave is more resistant to the suppressing action of topically applied KCl than is the N-wave of the primary complex. If the suppressing action manifests itself as KCl gradually diffuses from the surface to the deep layers, it is suggested that the SN-wave originates from a deeper layer of the cortex than does the N-wave. This is well supported by the intracortical recording of the evoked potential complex.

Fig. 8 shows the responses recorded from various depths beneath the cortical surface. As the microelectrode was progressively inserted into the depth of the

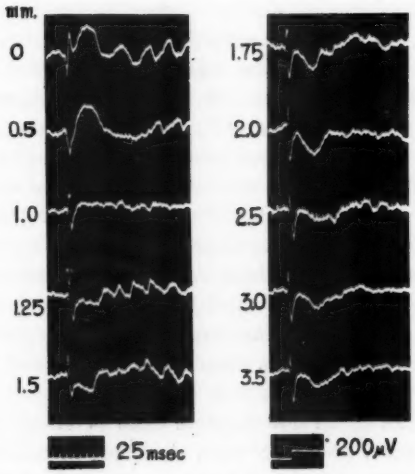

Fig. 8. Potential distribution in different depths of the cortex. Reversal point for SN-wave is located more deeply than for primary complex.

cortex, the first to be noted was the phase reversal of the primary complex. In this experiment the primary complex completed the reversal of phase before the depth of 1.0 mm was reached. This level was found to be the isoelectric point for the SN-wave. At 1.25 mm beneath the surface the reversed SN-wave, which is positive in sign, became evident. The depth at which the phase reversal of each component of the evoked potential occurred varied from case to case, but it was a consistent finding that the reversal point of the SN-wave was located at a deeper layer than in the case of the primary complex. The deep slow wave of positive polarity attained the maximal amplitude about 2 mm below the surface, and then it decreased gradually when the microelectrode was thrust more deeply.

The fact that the phase reversal of the SN-wave took place below the cortical surface suggests that it is an electrical potential localized within the cortex, and also that this potential is produced on the cortical neurones postsynaptically by thalamic afferent volleys.

DISCUSSION

In the present study it has been shown that the SN-wave is different from the primary complex in such points as threshold (Fig. 1), pharmacological

selectivity (Figs. 5, 6, & 7), intracortical potential distribution (Fig. 8) and so on. All these findings indicate clearly that the SN-wave is a separate process from the primary complex and originates from some structures of a cortical layer deeper than that containing the element responsible for the N-wave.

When an extensive study was made by Chang[4] on the direct cortical response (DCR), he noticed that a slow negative wave followed a primary negative wave of short duration in response to a strong stimulus. According to Chang, the slow negative wave, called "the second component" of the DCR, had a higher threshold than the primary negative wave and it did not reverse its polarity when recorded from a deep layer 1 mm below the cortical surface. In contrary to Chang, Goldring and his associates[5-7] reported the slow negative wave of the DCR to reverse its polarity in the deeper layers of the cortex. A similar slow negative wave was also found by these workers as a component of the recruiting response of the cerebral cortex to repetitive stimulation of the thalamic intralaminar system. According to these workers, GABA, applied to the cortical surface, augments the slow negative wave in the DCR as well as in the recruiting response.

The characteristics of the slow negative wave as described by Goldring *et al.* well coincide with those of the SN-wave described in this paper, a suggestion being made that both are generated by the same mechanism. However, there seems some discrepancy between the slow wave of the DCR and the SN-wave of the primary evoked potential in regard to the intensity of stimulus required to elicit them. It has been mentioned in the previous section that the threshold of thalamic stimulation is lower for the SN-wave than for the primary positive-negative complex, while it has been reported by Chang and by Goldring *et al.* that in the DCR a stronger stimulus is required for the slow negative wave than for the primary negative wave. This discrepancy may be explained that direct stimulation of the cortical surface is a more favorable method of activation for the primary negative wave than for the slow negative wave, because the latter is considered to originate from the layer slightly deeper than those generating the former.

Discussion on the mechanism of potential generation of the SN-wave is rather a difficult problem. We have shown that the SN-wave from the surface and the nearby superficial layers is associated with the slow wave of positive polarity in the deeper layers. This fact may indicate that there is an electric dipole which has the sink in the superficial layers against the source of the deeper layers. However, a question still remains to be answered which one, the superficial negativity or the deep positivity, is the locally produced active process. If the deep positivity represents an active process, the superficial negativity may be a passive phenomenon which is caused by spread of the current from the source in the deeper layers. If one follows this notion, the experimental finding

that surface-applied GABA augments the superficial negativity and the deep positivity simultaneously must be explained as follows. Though GABA is placed on the cortical surface, it penetrates into the deeper layers and enhances the electrical activity of positive polarity arising therefrom, and in proportion to the enhancement of the deep positivity, the negativity of the surface and superficial layers will also be increased. However, this cannot be accepted in view of the finding of Yamamoto *et al.*[8] obtained with the cat cerebral cortex. They showed that if surface-applied GABA was maintained in a low concentration, its action was limited to the superficial layers within 1 mm below the surface. This may also be true of the dog cerebral cortex studied in the present experiment. Therefore, the GABA-induced augmentation of the SN-wave must be ascribed to enhanced activity of the nervous elements lying in the superficial layers where the SN-wave is recorded with the negative polarity. This means that the enlargement of the deep positivity under the action of GABA is an event caused passively by the augmentation of the superficial negativity. This consideration will lead us to an inference that in the normal intact cortex, the SN-wave of negative polarity originates as locally produced activity in the superficial layers, causing an electrotonically spread potential of positive polarity in the deeper layers.

The above-stated consideration is obviously based upon the assumption that GABA has an excitatory action upon some types of the electrical activity of the cerebral cortex. This seems not in agreement with the well-accepted notion that the action of GABA is an inhibitory nature. In recent times, however, some authors have suggested that the action of topically applied GABA on the cerebral cortex cannot be explained only in terms of inhibition.[9-11] Moreover, as noticed by Iwama and Jasper under certain experimental conditions, a powerful enhancement of the sensory after-discharges followed local treatment of the cortical surface with GABA. In the present study it was occasionally observed that the sensory after-discharges, probably identical with the fast after-discharges of the intact cortex in Bremer's classification,[12] were enhanced extremely by local treatment with GABA and sometimes it appeared like the seizure wave. Thus, it is probable that GABA does not always exert an inhibitory action on the cerebral cortex, at least in the non-anesthetized state.

SUMMARY

The cerebral cortex of curarized, non-anesthetized dogs responded to a single stimulation of the thalamic somatosensory nucleus with an evoked potential consisting of a primary positive-negative diphasic complex (P- and N-waves) followed by a slow negative wave (SN-wave) of 100-150 msec in duration. The properties of the SN-wave were studied in comparison with those of the P- and the N-waves.

1. The localization of the SN-wave was limited within the somatosensory

cortex, and its threshold was slightly lower than that of the primary complex. When the thalamic stimuli were applied at an appropriate frequency, the SN-wave responded in a waxing and waning fashion.

2. In light barbiturate anesthesia, the SN-wave became unstable more readily than did the primary complex. By high frequency stimulation of the midbrain reticular formation the P-wave was more or less suppressed, but the effects on the N- and the SN-waves were quite variable.

3. The SN-wave was more resistant to systemic asphyxia or topically applied KCl than was the N-wave. Strychnine augmented the primary complex, especially its N-wave, whereas the SN-wave was little affected. By topical treatment with GABA the N-wave was abolished and the SN-wave was extremely enhanced.

4. The intracortical recording revealed that the SN-wave originated from some deeper points of the cortex than those producing the primary complex.

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Electromyographic Studies on Urinary Tract
IV. The Effects of Muscular Relaxants on the EMG of the Human and Canine Ureters

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INTRODUCTION

With 14 patients and 50 adult dogs, and using the recording method of the ureteral EMG referred in our 1st report[1], the effects of the two muscular relaxants, succinylcholine chloride (SCC) and d-tubocurarine chloride (d-Tc), on the ureteral smooth muscle were observed. The ureteral EMG and the EMG of the respiratory muscles which belong to the striated muscles were recorded simultaneously. We obtained a few interesting findings.

EXPERIMENTAL METHODS

1) 14 patients with intestinal diseases and seemingly healthy in their urinary organs were laparotomized under endotracheal anesthesia, and the ureters were exposed. Then bipolar (electrode distance: 1–3 cm) or unipolar leading was made before the scheduled operation.

2) The experimental dogs (6–20 kg) were fixed in dorsal position in a laboratory 15–25°C in temperature, then laparotomized under i.v. anesthesia with thiopental sodium. The ureteral EMG was recorded by bipolar leading (electrode distance : 3 cm).

For recording of the EMG of smooth muscle, a four stages amplifier (one CR-coupled and three direct, time constant: 2.5 sec.), Sanei & Co. or a Poly-Viso, Sanborn & Co. were used. Silver needle electrodes were used. For bipolar leading, one electrode was located at the proximal part and the other at the distal part of the ureter. For unipolar leading, the different electrode was located on the ureter and the indifferent one in the fatty tissue around the ureter.

For recording of the EMG of striated muscles, a CR four stages amplifier (time constant: 0.05 sec.), Tokyo Denki Seiki & Co. and as electrode a coaxial needle electrode were used. The EMG was led from the external intercostal muscle.

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For recording of the EMG, the heat writing recorder attached to the Poly-Viso or an H-type vibrator of Yokokawa's electromagnetic oscillograph was used.

As the experimental drugs, the two muscular relaxants SCC and d-Tc were used.

These drugs were administered intravenously to the patients under assisted respiration or controlled respiration and in the cases of dogs, under artificial respiration with automatic negative-positive respiration. As the usual dose, SCC 1 mg/kg, d-Tc 0.1 mg/kg, and in the cases of dogs, SCC 10 mg/kg., and d-Tc 1 mg/kg. were used as the larger doses.

Reading of the ureteral EMG: The classification of wave patterns and measuring of frequency, amplitude, duration and propagation velocity were the same as described in the 1st report[1].

The frequency of the waves shows variation according to the lapse of the time as we have already pointed out; therefore, the author decided that it showed an increase or a decrease in frequency when it varied over or below 3 w/min. after the administration of the drugs.

EXPERIMENTAL RESULTS

I. EMG of the human ureter

1) Findings after i.v. administration of SCC (8 cases)

In all the cases after i.v. administration of SCC in the usual dose of 1 mg/kg., the respiratory movement stopped about 1 min. after the injection and recovered about 3 min. thereafter. The EMG before and after the injection showed nearly the same figures in all the 8 cases.

The frequency of the waves was 1–5 w/min. before and 1–4 w/min. after the injection, thus no marked difference in frequency was found before and after the administration of SCC in the usual dose, as shown in Fig. 1.

The wave pattern showed no change by the injection (Fig. 2), and the amplitude was 0.8–1.4 mV before and 0.8–1.5 mV after the injection and the duration of the waves stood at the same value of 0.23–0.59 sec. before and after the injection. The propagation velocity of the waves was 18.0–27.7 mm/sec. before and 19.0–27.7 mm/sec. after the injection, showing no marked change.

In a case in which the usual dose of SCC was administered three times at the interval of 10 min., no marked change on the EMG was observed.

2) Findings after i.v. administration of d-Tc (6 cases)

In all the cases injected with the usual dose of d-Tc 0.1 mg/kg., the respiratory movement diminished or disappeared about 1 min. and 30 sec. after the injection and recovered about 15 min. thereafter. Electromyographically, the wave frequency (1–6 w/min. before and after the injection) and the wave pattern showed

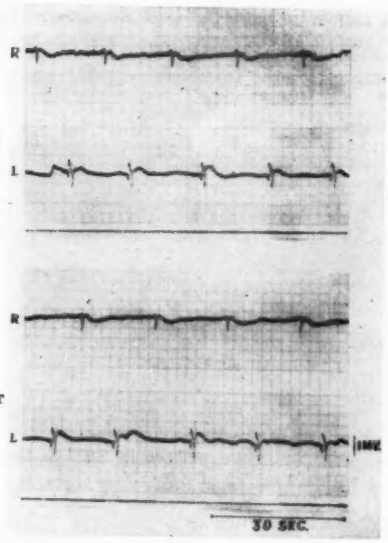

Fig. 1. Frequency in EMG of the human ureter by i.v. injection with 1 mg/kg. of SCC.

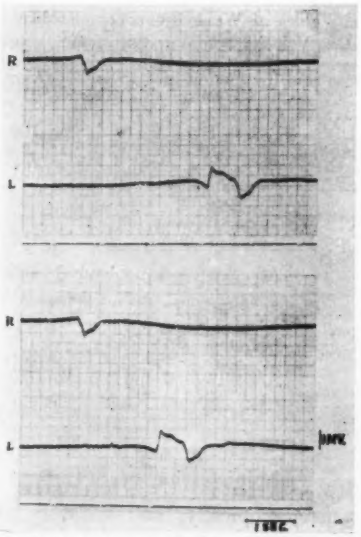

Fig. 2. Wave pattern in EMG of human ureter in the above case.

no marked change as shown in Fig. 3. The amplitude, the duration and the propagation velocity showed the same values of 0.8–1.3 mV., 0.2–0.4 sec. and 15–26 mm/sec. before and after the injection, respectively.

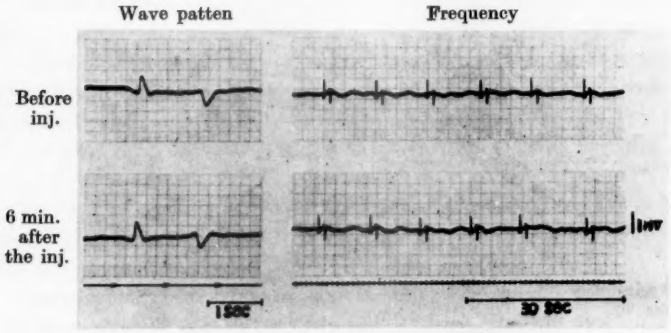

Fig. 3. Frequency and wave pattern in EMG of human ureter by the i.v. injection with 0.1 mg/kg of d-Tc.

Thus in the ureteral function no significant change was observed by the administration of the SCC or d-Tc in the usual dose, though they were effective in inhibiting or stopping the respiratory movement (Tables I and II).

TABLE. I. Measured Values of Wave Frequency, Pattern, Amplitude, Duration and Propagation Velocity before and after the Injection of S.C.C.

	Number of Case	Dose of SCC	Frequency in w/min		Wave Pattern	Amplitude (mV)	Duration (sec)	Velocity (mm/sec)
Human	8	1 mg/kg	Bef. 1-5			0.8-1.4	0.23-0.59	18.0-27.7
			Aft. 1-4		unchanged	0.8-1.5	0.23-0.59	19.0-27.7
Dogs	18	1 mg/kg	15 cases	3 cases				
			Bef. 2-8	2-5		0.5-1.8	0.21-0.48	20.0-39.4
			Aft. 2-9	5-8	unchanged	0.5-1.8	0.20-0.47	21.2-40.5
	10	10 mg/kg	9 cases	1 case				
			Bef. 1-6	5		0.3-4.0	0.26-0.51	21.7-39.4
			Aft. 4-15	6	unchanged	0.3-4.0	0.24-0.49	19.5-38.5

II. EMG of the dog's ureter

1) Findings after i.v. administration of SCC.

In all the 18 cases, macroscopically, the respiratory movement stopped about 30 sec. –1 min. after the injection of the normal dose of SCC 1 mg/kg. and did not recover within 20 min. after the injection.

TABLE II. Measured Values of Wave Frequency, Pattern, Amplitude, Duration and Propagation Velocity before and after the Injection of d-Tc.

	Numder of Case	Dose of d-Tc	Frequency in w/min		Wave Pattern	Amplitude (mV)	Duration (sec)	Velocity (mm/sce)
Human	6	0.1 mg/kg	Bef. 1-6			0.8-1.3	0.20-0.41	15.0-26.0
			Aft. 1-6		unchanged	0.8-1.3	0.20-0.41	15.0-26.0
Dogs	12	0.1 mg/kg	10 cases	2 cases				
			Bef. 2-7	2-9		0.7-1.7	0.19-0.35	22.8-34.1
			Aft. 1-6	0-5	unchanged	0.6-1.8	0.18-0.36	22.5-34.5
	10	1 mg/kg	8 cases	2 cases				
			Bef. 2-9	6-9		0.5-1.4	0.20-0.45	22.0-36.4
			Aft. 0.4	9-13 / 0-1	unchanged	0.5-1.4	0.20-0.45	21.5-38.4

In 15 of the 18 cases, the frequency of the waves was 2–8 w/min. before and 2–9 w/min. after the injection, showing no significant change after the injection of SCC in a usual dose (Fig. 4). In the other 3 cases, it rose from 2–5 w/min. before to 5–8 w/min. after the injection, showing an increase in frequency, and then it recovered 3–5 min. after the injection. The wave pattern showed no change with the administration of this drug in all the cases (Fig. 5), and the

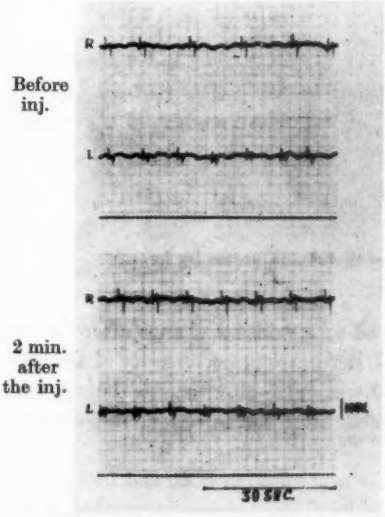

Fig. 4. Frequency in EMG of canine ureter by the i.v. injection with 1 mg/kg of S.C.C.

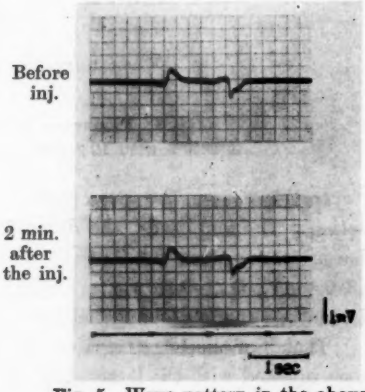

Fig. 5. Wave pattern in the above case.

amplitude, the duration and the propagation velocity were 0.5–1.8 mV., 0.21–0.48 sec., 20.0–39.4 mm/sec. before and 0.5–1.8 mV., 0.20–0.47 sec., 21.2–40.5 mm/sec. after the injection, respectively, showing no significant change by the injection.

In the 10 cases, when a larger doses of SCC 10 mg/kg. was injected intravenously, the respiratory movement stopped within 20–50 sec. after the injection and did not recover within 30 min.

In 9 of the 10 cases, the frequency of the waves was 1–6 w/min. before and 4–15 w/min. after the injection, showing a marked increase, however, normalized 2–6 min. after the injection (Fig. 6). In the other one case, it was 5 w/min. before and 6 w/min. after the injection.

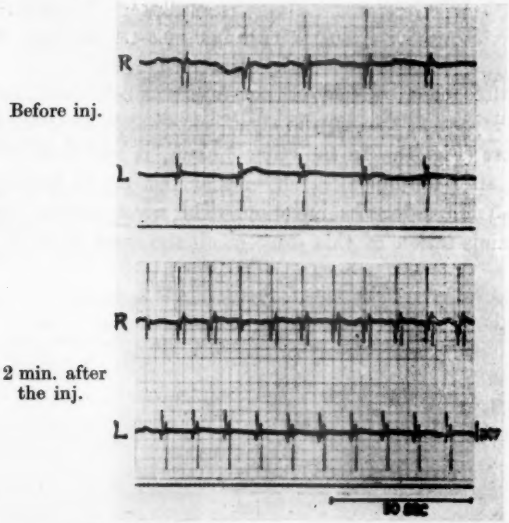

Fig. 6. Frequency in EMG of the canine ureter by i.v. injection with 10 mg/kg. of SCC.

In these cases, the wave pattern showed no significant change after the injection. The amplitude, the duration and the propagation velocity of the discharge waves were 0.3–0.4 mV., 0.26–0.51 sec., 21.7–39.4 mm/sec. before and 0.3–0.4 mV., 0.24–0.49 sec., 19.5–38.5 mm/sec. after the injection, respectively, showing no marked change, either.

That 3 of the 18 cases injected with the usual dose of SCC, and 9 of the 10 cases injected with the larger dose of SCC showed an increase in number of the peristaltic discharges (Table I) seemed to indicate that SCC accelerates the peristaltic movement of the dog's ureter.

2) Findings after i.v. administration of d-Tc

When the usual dose of d-Tc 0.1 mg/kg. was administered intravenously in 9 of the 12 cases, the respiratory movement was inhibited and in the other 3 cases, it was stopped 42 sec.–1 min., 30 sec. after the injection but recovered 3–12 min. after the injection, macroscopically.

Electromyographically it was observed that in 10 of the 12 cases, the frequency was 2–7 w/min. before and 1–6 w/min. after the injection, showing no marked change (Fig. 7).

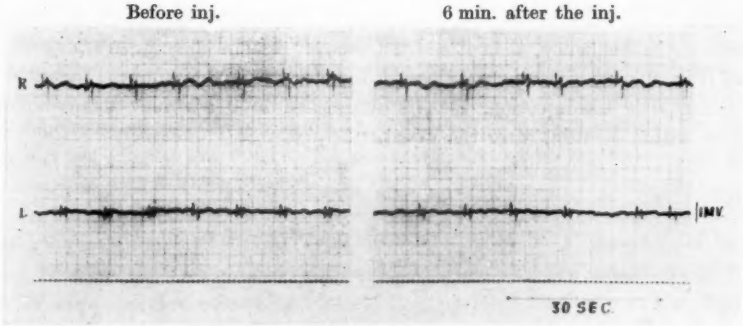

Fig. 7. Frequency in EMG of canine ureter after i.v. injection with 0.1 mg/kg. of d-Tc.

In the other 2 cases, it was 2–9 w/min. before and 0–5 w/min. after the injection, showing a decrease in frequency, then it recovered 7–10 min. after the injection. In these 12 cases, the wave pattern showed no significant change by

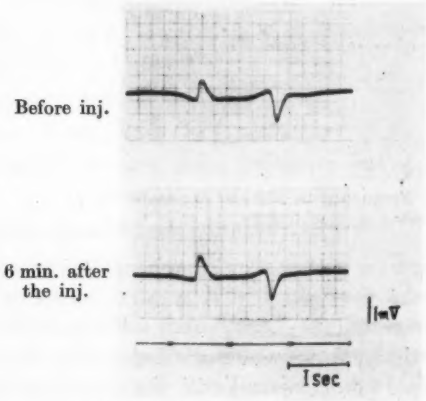

Fig. 8. Wave pattern in the above case.

the injection (Fig. 8). Besides, the amplitude, the duration and the propagation velocity of the waves were 0.7–1.7 mV., 0.19–0.35 sec. and 22.8–34.1 mm/sec. before and 0.6–1.8 mV., 0.18–0.36 sec. and 22.5–34.5 mm/sec. after the injection, respectively, showing no marked change by the injection of the larger doses of d-Tc 1 mg/kg.

In the 10 cases, macroscopically, the respiratory movement was found stopping 30 sec. – 1 min. after the intravenous administration of d-Tc in a dose of 1 mg/kg., and not recovering within 30 min. after the injection.

On the electromyographical examination, in 8 of the 10 cases the frequency registered 2–9 w/min. before and 0–4 w/min. 3–6 min. after the injection, showing a decrease in frequency, then it did not recover within 30 min. in many of the cases (Fig. 9). In the other 2 cases, it increases from 6–9 w/min. before to 9–13 w/min. after the injection, then it diminished to 0–1 w/min. and it did not recover within 30 min. after the injection.

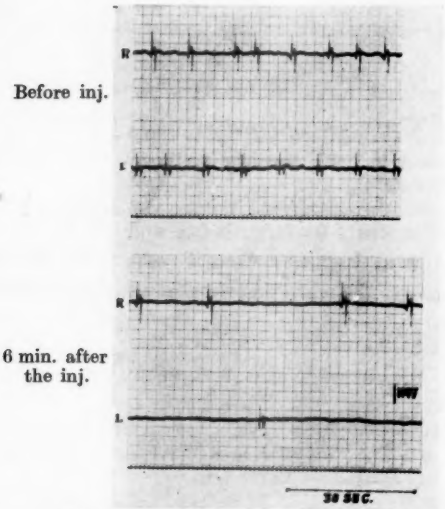

Fig. 9. Frequency in EMG of canine ureter by i.v. injection with 1 mg/kg. of d-Tc.

In all the 10 cases, the wave pattern showed unchanged forms, and the amplitude, the duration and the propagation velocity were 0.5–1.4 mV., 0.20–0.45 sec., 22.0–36.4 mm/sec. before and 0.5–1.4 mV., 0.20–0.45 sec., 21.5–38.4 mm/sec. after the injection, respectively, showing no marked change by the injection.

In short, that 2 of the 12 cases injected with the usual dose of d-Tc and 8 of the 10 cases injected with the larger doses of d-Tc showed a decrease in

frequency seemed to indicate that d-Tc acts inhibitory on the peristaltic movement of the ureter (Table II).

III. EMGs of the external intercostal and the ureteral muscles in dogs (10 dogs)

The EMGs of the external intercostal muscle and of the ureter were recorded simultaneously.

1) Findings after the i.v. administration of SCC

In the 2 cases, before the injection of SCC, dense respiratory discharges with the amplitude of 100–150 μV. were observed. They decreased or disappeared 30–60 min. after the injection. Before the disappearance, denser discharges with the amplitude of 100–250 μV. were observed simultaneously with marked dyspnea.

Electromyographically speaking, the frequency, the wave pattern, the duration and the propagation velocity in the ureter showed no marked change after the injection with SCC 1 mg/kg.

In the other 3 cases, dense respiratory discharges with the amplitude of 100–150 μV disappeared 20–40 sec. after the injection of SCC 10 mg/kg. of larger doses. Before the disappearance denser discharges with the amplitude of 100–250 μV were observed coinciding with marked dyspnea, as shown in Fig. 10.

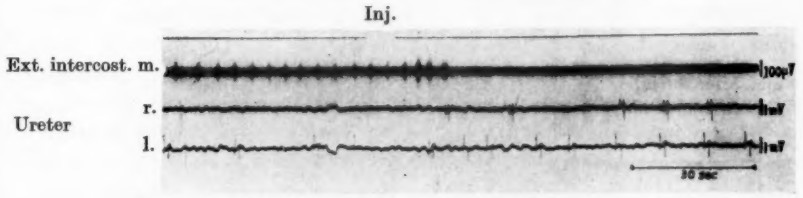

Fig. 10. In the above case, the administered drugs were increased to 10 mg/kg.

In the ureteral EMG, the peristaltic discharges showed an increase in frequency 25 sec. –1 min. after the injection, but the wave pattern, the duration and the propagation velocity of the discharges showed no marked change by the injection.

2) Findings after i.v. administration of d-Tc

In the 2 cases, as shown in Fig. 11, before the injection, dense respiratory discharges with the amplitude of 150–200 μV. were found. They showed a slight decrease in frequency, when the amplitude was 100–200 μV, 40 sec. –1 min. after the injection of d-Tc 0.1 mg/kg., but in the ureteral EMG, the frequency, the duration and the propagation velocity of the discharges showed no significant change as referred to above.

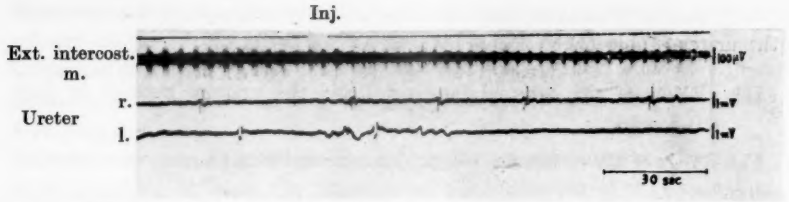

Fig. 11. Synchronous recording of EMG of external intercostal muscle and ureter by i.v. injection with 0.1 mg/kg of d-Tc.

In the other 3 cases, as shown in Fig. 12, before the injection of d-Tc, dense respiratory discharges with the amplitude of 150–250 μV were observed. They disappeared 30 sec. –1 min. after the injection of d-Tc 1 mg/kg.

In the ureteral EMG, the peristaltic discharges showed a decrease in frequency or disappearance 3–6 min. after the injection, but the wave pattern, the duration

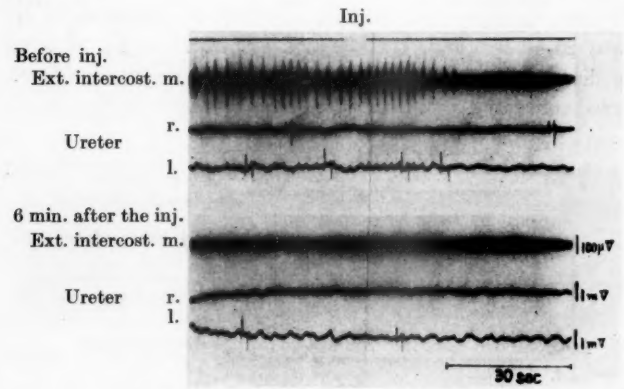

Fig. 12. EMG of external intercostal muscle and ureter by i.v. injection with 1 mg/kg. of d-Tc.

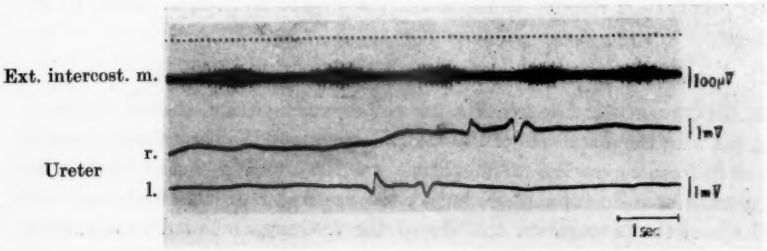

Fig. 13. EMG of external intercostal muscle and bilateral ureters.

and the propagation velocity of the discharges showed no marked change by the injection.

In recording of the both respiratory and the ureteral EMG, when the paper moved slowly as shown in the above figures the detail of the wave pattern could not be observed clearly, but when it moved more rapidly the pattern became more clearly, as shown in Fig. 13.

DISCUSSION

It is an interesting problem how SCC and d-Tc, the useful muscular relaxants, affect the smooth muscles, but studies of the effects of the muscular relaxants on such muscles were quite fewer than those on the striated muscles.

Gross[2] has reported on the effect of curare on the intestines; he mentioned that after the i.v. administration of d-Tc in a dose of 1–1.5 mg/kg., the canine small intestine became atonic and the peristaltic movement in it disappeared. On the other hand, Swanson[3] reported that the i.v. administration of d-Tc in a dose of 10–15 mg/kg. caused no change in the peristaltic movement of the canine intestine. Thesleef[4] reported that a dose of 0.3–1.0 mg/kg. (1–3 times the d-Tc sufficient to paralyse the striated muscle) was required to block off the inferior mesenterial ganglion, but Everett[5] reported that the administration of d-Tc in a dose of 0.25–0.5 mg caused an acceleration of the peristaltic movement of the rabbit's excised intenstines, and Ikeda[6] reported that the administration of curare in a dose of 10 mg. caused an increase in tonus in the excised rabbit's intestines.

On the effect of curare on the ureter, Lapides[7] reported that when unanesthetised patients who had probably intact urinary organs were injected with 120 units of intocostrin, all striated muscles except in the diaphragma were paralysed though the peristaltic movement of the ureter showed no change. Harris[8] reported a clinical case in which the peristaltic movement was observed even after the administration of d-Tc in a dose which did not cause anoxia, while Butcher[9] reported that when d-Tc 5 mg/kg. was injected i.v. and in the ureteral wall in a dose of 1 mg., the EMG of the dog's ureter showed no change.

From our experimental results, it was concluded that a usual dose of d-Tc causes no marked change in the peristaltic movements of human and canine ureters, but that a larger dose of d-Tc causes inhibition of the peristaltic movement of the dog's ureter, in accordance with the experimental results of the other authors.

On the effect of SCC on the ureteral function, no report has appeared.

CONCLUSION

1) Electromyographically, in human subjects, the i.v. administration of SCC in a dose of 1 mg/kg. and d-Tc in a dose of 0.1 mg/kg. caused no change in

the ureteral peristaltic movement.

2) The i.v. administration of SCC in the usual dose of 1 mg/kg. caused no marked change in the frequency in 15 of the 18 cases, but in the other 3 cases the frequency increased.

In all the 18 cases, the wave pattern, the duration, the amplitude and the propagation velocity showed no significant change after the injection.

The i.v. administration of SCC 10 mg/kg. caused an increase in frequency in 9 of the 10 cases, and no change in the other 1 case, but caused no significant change in the wave pattern, the duration, the amplitude and the propagation velocity of the discharges.

3) The i.v. administration of d-Tc 0.1 mg/kg. in 10 of the 12 cases, caused no marked change, and in the other 2 cases caused a decrease in frequency.

The i.v. administration 1.0 mg/kg. of a larger dose caused a decrease in frequency in 8 of the 10 cases, and a decrease after temporary increase in the other 2 cases while in the wave pattern, the amplitude, the duration and the propagation velocity no marked change was found in each of the cases.

4) The simultaneous recording of the EMG of the respiratory muscles and of the ureter showed that the i.v. administration of SCC 1 mg/kg. and d-Tc 0.1 mg/kg. caused the disappearance or the diminution of the respiratory discharges, but caused no marked change in the ureteral peristaltic discharges.

The i.v. administration of both SCC and d-Tc in larger doses, 10 mg/kg. and 1 mg/kg., respectively, caused the disappearance of the respiratory discharges, but on the ureteral EMG, the former caused an increase and the latter caused a decrease in wave frequency.

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Studies on Somatotropic Hormone Secretion in Gynecology and Obstetrics

By

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The statement that the placenta produces its own gonadotropin, estrogen, progesteron and corticoid, has been generally accepted. Recently prolactin, ACTH- and TSH-like hormone productions have also been attributed to it, but the existence of somatotropic hormone (=STH), a hormone that has come into attention only very recently, in the placenta has been suspected but not yet definitively demonstrated.

It has been ascertained by much research that STH is secreted from the anterior pituitary lobe, in particular, from the eosinophilic cells (α cells). But the questions of whether it can be extracted from other tissue or humor, how its content is, if present in such organs and tissues, how it is affected by the physiologically specific condition of pregnancy, and what is the physiological significance of such a change of the content are yet unanswered. In order to clarify these problems, the author tried extraction of STH-like hormone from human placenta of the early, middle and later stages of pregnancy, the plasma of pregnant and post-puerperal women, umbilical cord blood, amniotic fluid and urine of newborns, as well as from the plasma of a female dwarf with diabetes mellitus.

EXPERIMENTAL METHODS

[A] Extraction of STH-like substance from placental tissue

A method similar to that used by Wilhelmi *et al.* in making the extract from the pituitary gland was employed. All of the steps are outlined in Table I. They were carried out at 1°C.

Placental tissue was minced with a masticator, homogenized, and filtrated with aceton. The precipitation was stirred vigorously with $Ca(OH)_2$ solution at pH 10.3 for about one hour and was then kept frozen for 6 hours. After being thawed, the mixture was centrifuged in Spinco. L-shaped ultracentrifuge at a speed of 20, 000 RPM, and the residue washed with $Ca(OH)_2$ solution at pH 10.3. The supernatant fluid and the washing were combined and brought to 0.5 satura-

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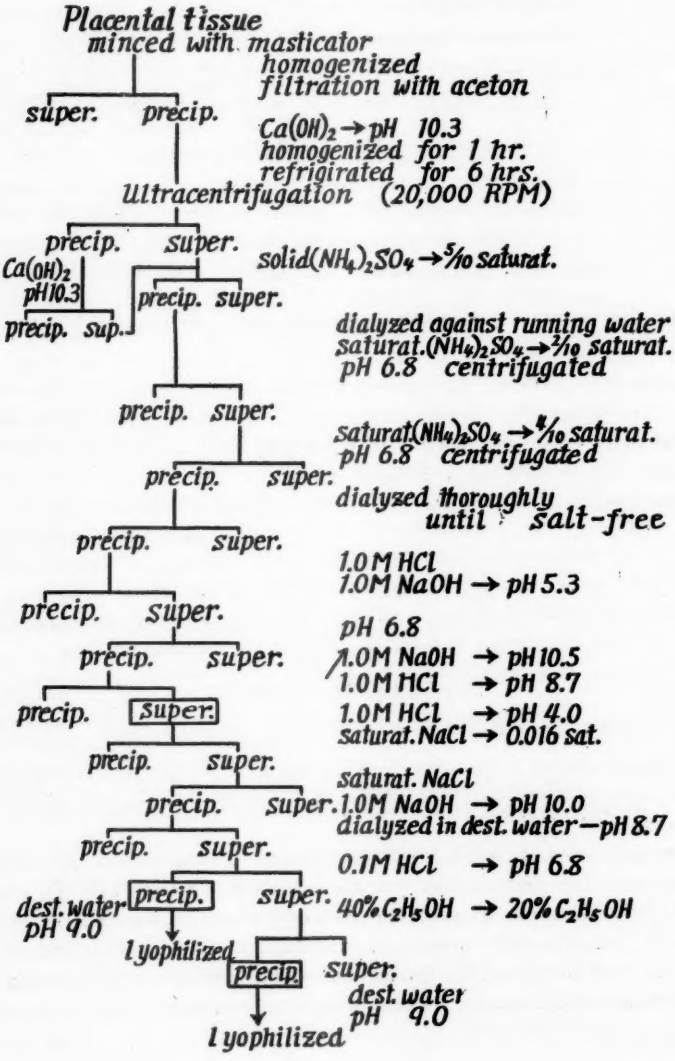
tion, with respect to ammonium sulfate, by the addition of solid $(NH_4)_2SO_4$. The precipitate formed was dissolved in 200 cc of water and dialyzed against running water for 16 to 20 hours. After dialysis, a saturated ammonium sulfate solution was added until the concentration of $(NH_4)_2SO_4$ was brought to 0.2 saturation,

TABLE I. Method of Extraction from Placenta.



and the mixture was adjusted to 6.8. The precipitate formed was centrifuged and discarded. The supernatant fluid was brought to 0.4 saturation by the addition of more saturated ammonium sulfate at pH 6.8, and the precipitate formed was dissolved in water and dialyzed thoroughly until salt-free. The insoluble material inside the dialysis bag was dissolved in water with the aid of 1.0 M HCl. After pH-fractionation as shown in Table I, a saturated solution of NaCl was added dropwise to 0.016 saturation. To the supernatant fluid an equal volume of saturated NaCl was added slowly. The precipitate was dissolved in water adjusted to pH 10.0, and dialyzed against distilled water. The dialyzed solution was adjusted to pH 8.7. The pH of the supernatant was adjusted to 6.8. After centrifugation, the precipitate was dissolved in water adjusted to pH 9.0 and lyophilized, but the supernatant at pH 6.8 was next brought to an ethanol concentration of 20% by adding very slowly an equal volume of 40% ethanol. The precipitate was dissolved in water adjusted to pH 9.0 and the solution was then frozen and dried in a vacuum.

[B] Extraction of STH-like substance from blood, amniotic water and urine

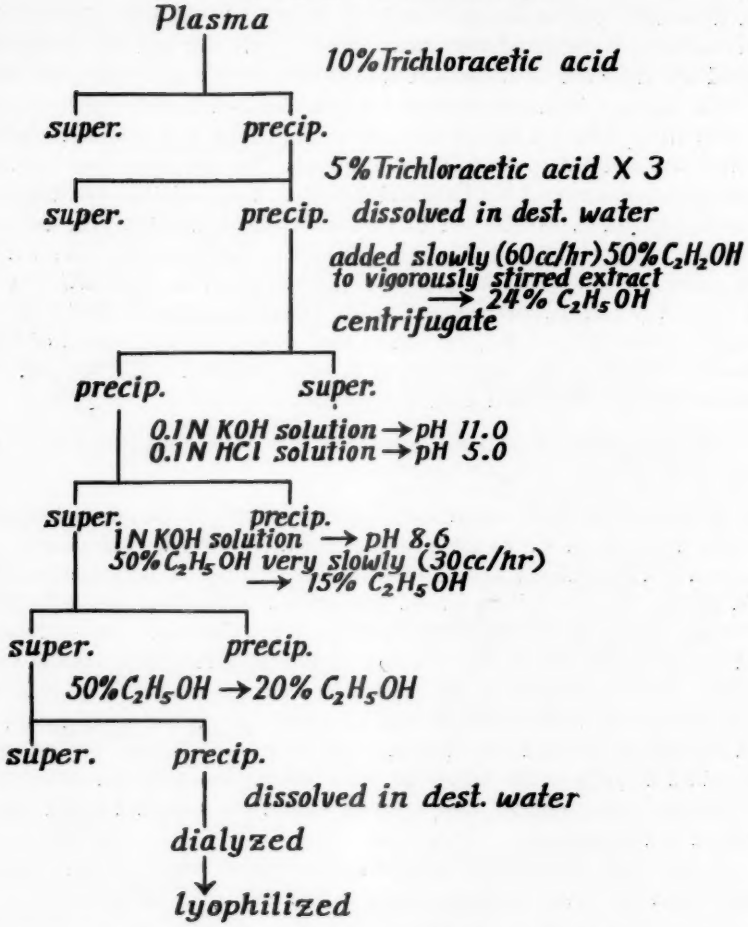
As shown in Table II, a modification of the Gemzell's method was attempted. Freshly drawn blood was cooled in ice-water and centrifuged. The plasma was removed and immediately treated with the same volume of 10% trichloracetic acid (TCA). The precipitate formed was washed three times with 5% TCA. After the last centrifugation the TCA precipitate was dissolved in distilled water and brought to an ethanol concentration of 24% by adding 50% ethanol very slowly. After centrifugation, the precipitation was dissolved with 0.1 N KOH solution at pH 11.0. Whenever precipitation occurred at pH 5.0, it was discarded, and the supernatant fluid was adjusted to pH 8.6 with 1 N KOH. 50% ethanol was added dropwise to the fluid while being stirred vigorously and cooled to a 15% ethanol concentration. The precipitate formed was suspended in cold water, dialyzed and lyophilized.

A light-brown or light-yellow powdery substance, which showed positive Biurett and Ninhydrin reactions, was obtained only from plasma.

[C] Biological determination of STH-like substance

The author compared five methods which have been suggested as better assay procedures using the same purified hormone preparation. They are 1) assay based on the rate of blood urea nitrogen formation after the injection of an enzymatic casein hydrolysate in nephrectomized rats, 2) tibia test, 3) assay based on the formation and calcification of the dentine recorded by the lead acetate vital staining method, 4) assay based on the increased rate in body weight and 5) assay based on the increased rate in tail length. The details of these

TABLE II. Method of Extraction from Plasma



methods and results have been given in a previous report[1]. The comparison of the five methods is summarized in Table III and from these viewpoints of the sensitivity, the specificity, the duration of treatment and ease of the technique, the tibia test is considered to be more advantageous.

The question of specificity has been repeatedly discussed[2)-5)], but it has been confirmed that non-STH agents will not broaden the width of the epiphyseal cartilage over 20–30 μ, even by further increased doses.

The Tibia Test: The extract was injected intramuscularly once daily for

TABLE III. The Comparison of Assay Methods of STH

Mothods**	Duration (days)	Sensitivity (mg)	Range of 99% reliable limit (ρ)	Correlation coefficient (T)
I	3/4	0.1	0.73	0.92
II	5	0.01	0.91	0.96
III	10	0.01	1.21	0.94
IV	15	0.75	4.00	0.68

* Minimum amount of STH to produce a significant response.
** I method is based on the rate of blood urea-N.
II method is tabia test.
III method is based on the formation of dentine.
IV method is based on the rate of body weight.

four days. Twenty four hours after the last injection the rats were sacrified under ether anesthesia, immediately the proximal end of the tibia was split in the midsagittal plane. After the bone halves were treated with aceton and silver nitrate, the width of the uncalcified portion was measured under low power using a micrometer.

The responses, which were obtained when graded doses of the standard STH were administered, show a straight line when plotted against the logarithm of the doses. From these data it was calculated that the positive correlation with the coefficient $\gamma=0.96$ was substantiated. The equation of the regression line is shown as follows: (Table IV, Figure 1).

$\log Y=0.019X-3.66\pm0.91$

X: The width of the proximal epiphyseal cartilage of the tibia (μ)
Y: Total doses of STH (mg)

The biological activity of the extracted substances was measured with the aid of a standard curve constructed with increasing concentration of bovine STH (Armour lot. R. 50109).

TABLE IV. The Effect of STH on the Width of the Tibial Cartilage.

Groups	Total dose of STH (mg.)	No.	Width of tibial cartilage		Average (μ)
			right (μ)	left (μ)	
STH treated	10.0	3	255	225	240
	1.0	3	198	192	195
	0.5	3	191	147	169
	0.1	3	147	137	142
	0.01	3	101	93	97
Control		3	18	19	18

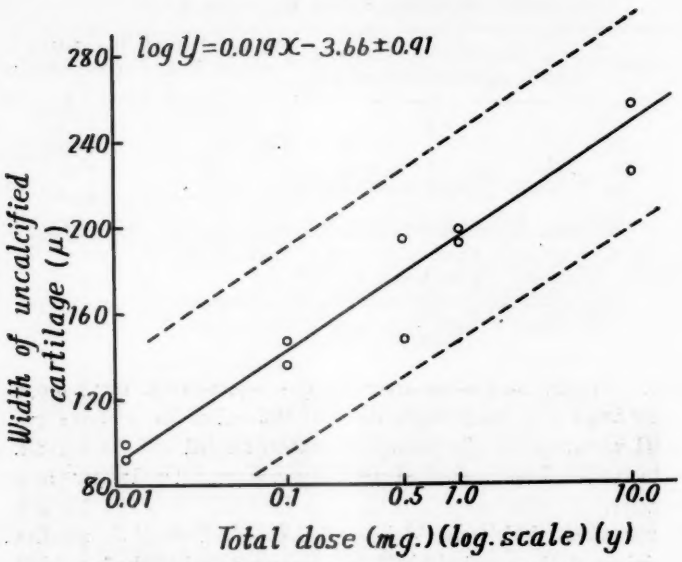


Fig. 1 Standard dose-response curve for the tibial cartilage response to STH.

EXPERIMENTAL RESULTS

[A] Recovery of added STH from human plasma

The standard STH (0.5 mg, 1.0 mg, and 10 mg) was dissolved in a small amount of physiologic saline and added slowly at 0°C with stirring to normal human plasma. It was immediately fractionated according to the method described. The active fractions were injected into the assay rats. As seen in Table V, the recovery varied between 84 and 100%. It is possible that the relatively high recovery of added STH may have been due to substances in plasma such as thyrotropic hormone which acted synergistically with STH, and which alone did not yield a positive result using the tibia test.

Table V. Recovery of added STH from Human Plasma.

Amt. of added STH (mg)	Amt. of protein in active fraction (mg)	Total dose of fractionated plasma per assay rat (mg)	No. of assay rat	Average width of tibial epiphysis (μ)	Recovery of added STH (%)
0.5	550	180	3	180± 4.2	100
1.0	260	80	3	165±12.4	84
10.0	550	180	3	240±12.0	95

[B] Concentration of STH-like substance in the plasma

The author tried extraction of STH-like hormone from human plasma of the early, middle and later stages of pregnancy, and from the plasma of post-puerperal women, a juvenile diabetic patient with retarded growth, and a normal woman as control. As indicated in Table VI, it can be seen that up to 10 weeks' gestation in each case the treated group did not show the mean width of epiphyseal cartilage exceeding 160 μ, which increased before the delivery and abruptly decreased after it. It is questionable whether these results may be regarded as reflecting the actual change in STH values in the respective stages of pregnancy. It is interesting to note that although no STH-like substance could be detected in the plasma of normal non-gravidae, it was found in the equivalent of 3 mg in a severe juvenile diabetic girl with retarded growth.

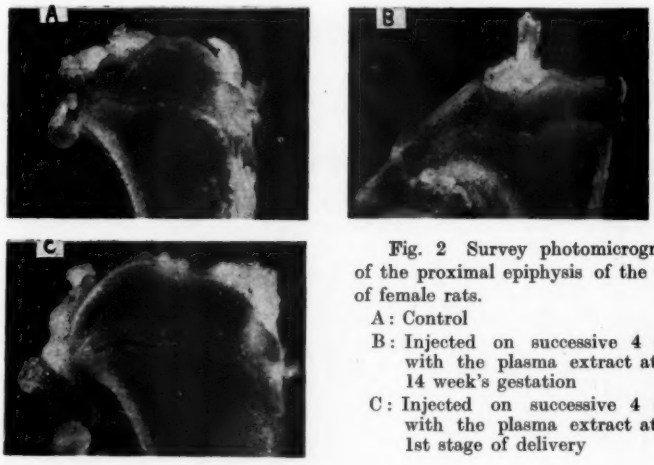


Fig. 2 Survey photomicrographs of the proximal epiphysis of the tibia of female rats.
A: Control
B: Injected on successive 4 days with the plasma extract at the 14 week's gestation
C: Injected on successive 4 days with the plasma extract at the 1st stage of delivery

[C] Concentration of STH-like substance in the placentae

Fresh placentae sampled at abortion or spontaneous delivery were immediately refrigerated and freed of blood, fetal membrance, etc. As the specimens from the earlier stages of pregnancy were too few for separate experimentation, the five specimens were subjected to a single process of extraction. In the early stage of pregnancy, the extract showed high efficacy in augmenting the width of the epiphyseal cartilage, but that extracted from the placentae of later pregnancy rarely caused the mean width to exceed 160 μ (Table VII, VIII and IX). There was no correlation between the weight of the placentae and the content of STH-like substance. Of the placentae from the full term pregnancy, specimens were selected from those accompanying a relatively heavy fetus, and we

TABLE VI. Concentration of STH in the Plasma.

Case					Amt. of plasma (ml)	Amt. of protein in active fraction (mg)	Total dose of fractionated plasma per assay rat (mg)	No. of assay rats	Average width of tibial epiphysis (μ)	Amt. of STH per 100 ml of plasma (mg)
No.	Initials	Age	Time of pregnancy	Body weight of newborn (g)						
3729	Y. W.	35	6 W.	—	60	250	83	3	159± 8.7*	—
C	K. S.	23	8 W.	—	60	55	18	3	113± 1.3	—
3984	S. O.	28	9 W.	—	65	220	73	3	83± 2.3	—
4132	K. S.	22	9 W.	—	38	20	6	3	92± 2.3	—
3649	M. T.	26	10 W.	—	70	120	40	3	143± 0.7	—
A	H. S.	21	14 W.	—	100	330	110	3	199±14.8	1.2
B	T. Y.	23	16 W.	—	100	620	206	3	202± 5.6	1.4
1000	M. Y.	31	1st stage of delivery	3040	70	1200	400	3	262± 1.9	42.5
976	N. K.	26	60 min. after deliv.	3520	65	1000	330	3	256± 3.8	35.3
661	H. T.	18	24 hrs. after deliv.	3790	60	1300	430	3	161± 6.8	—
601	N. S.	28	24 "	3300	60	800	260	3	180±19.0	0.8
220	Y. K.	24	24 "	4500	20	430	140	3	175± 7.2	1.9
838	Y. Y.	24	3 days after deliv.	3040	64	550	180	3	136±10.9	—
614	T. A.	22	4 "	3960	58	120	400	3	143±10.2	—
502	S. N.	34	4 "	2840	20	120	40	3	149± 1.7	—
497	T. S.	25	5 "	3620	20	260	80	3	147± 6.1	—
828	L. S.	29	9 "	3180	56	550	180	3	145± 2.1	—
1871	A. G.	18	hypoph. dwarf with diabates mellitus	—	60	1400	460	3	206±20.2	3.0
Cont.	M. F.	28	—	—	30	470	156	3	152± 3.4	—

* Mean ± standard error of the mean.

TABLE VII. Concentration of STH in the Human Placenta
[I] The Early Stage of Pregnancy.

No.	Time of pregnancy (w)	Amount of decidual tissue (g)	Amt. of protein in active fraction (mg)	No. of assay rats	Average width of tibial epiphysis (μ)	Amt. of STH per placenta (mg)
1	6	42	850	3	245±17.4	13.0
2	8			3	244± 8.9	12·5
3	5			3	235± 7.0	7.8
4	12			3	237± 5.2	8.6
5	12			3	233±16.7	7.0

TABLE VIII. Concentration of STH in the Human Placenta.
[II] The Middle Stage of Pregnancy.

Case				Weight of placenta (g)	No. of assay rats	Average width tibial epiphysis (μ)	Amt. of STH per placenta (mg)
No.	Initials	Age	Time of pregnancy (w)				
517	H. T.	27	20	170	3	190±12.5	0.8
623	M. S.	39	18	150	3	200± 6.3	1.3
634	T. E,	34	24	150	3	207±21.1	1.9

TABLE IX. Concentration of STH in the Human Placenta.
[III] The Full Term of Pregnancy.

Case					No. of assay rats	Average width of tibial epiphysis (μ)	Amt. of STH per placenta (mg)
No.	Initials	Age	Body weight of newborns (g)	Weight of placenta (g)			
220	Y. K.	24	4500	780	3	262±13.3	30.0
661	H. T.	18	3730	680	3	163±10.8	0.2
502	S. N.	34	3180	600	3	150± 2.0	—
497	T. S.	25	2840	500	3	126± 3.1	—
838	Y. Y.	24	3620	650	3	147± 1.4	—
614	T. A.	22	3960	630	3	160± 2.5	—
601	N. S.	28	3300	550	3	131± 3.3	—
Control (0.9 % saline)					3	145± 6.2	—

could not find any specific correlation between the weight of the fetus and the STH content.

[D] Concentration of STH-like substance in the umbilical cord blood, the amniotic water and the urine of newborns

The fresh umbilical cord blood collected immediately after childbirth was centrifuged. The plasma was fractionated and examined. The total dose of

the plasma fraction per assay rat did not exceed 200 mg. STH-like substance could be barely observed in the umbilical cord blood (Table X). Amniotic fluid was sampled with an amnion centesis-needle per peritoneum, and one day's urine of boys was collected with a urinator, but from neither STH could be isolated. It is apparently possible to detect STH-like substances in amniotic fluid only following their excretion in fetal urine, but first of all, it seems that a good method for extracting STH-like substances from urine is to be devised.

TABLE X. Concentration of STH in the Plasma of Umbilical Blood.

Case				Amt. of plasma (ml)	Amt. of protein in active fraction (mg)	Total dose of fractionated plasma per assay rat (mg)	No. of assay rat	Average width of tibial epiphysis (μ)	Amt. of STH per 100ml of plasma (mg)
No.	Initials	Age	Body weight of Newborn (g)						
450	Y. K.	27	3620	20	250	83	3	162± 2.1	0.95
540	S. K.	23	3100	20	330	110	3	158± 7.1	—
541	U. F.	29	2620	20	180	60	3	178±10.7	2.20
545	M. Y.	19	2140	20	200	65	3	195±11.2	5.00
Cont. (0.9 % saline)							3	159± 1.2	—

DISCUSSION

Many efforts have been made also for obtaining pure STH from the pituitary glands of cattle and pigs, and Li and Evans[6] (1945) and Wilhelmi *et al.*[7] (1948) succeeded in isolating nearly purified STH. Such extracted samples proved effective when administered to the respective animals, but not when applied to a human subject, owing to species resistance[8] and difference in STH sensitivity[9]. Li and Papkoff studied the hypophyseal STH from man, monkey and cattle, and pointed out the slight difference between them in biochemical properties[10]. So it is necessary to prepare the hormone from human hypophysis or other tissues for clinical experiments on human subjects. It is not at all difficult to surmise that STH is contained in blood, when we recall the results of experiments by Westman and Jacobson[11], who succeeded in discovering that the hypophysectomized one of parabiotic rats showed bodily growth. But a few authors have reported on success in extracting STH from blood by applying various chemical processes. Very recently, Gemzell has reported a commendable method[12] using ethanol fractionation technique showing a high rate of recovery.

On the subject of STH from the placenta, we have only the report by Ikeda *et al*[13]., who observed neogenesis of bone tissue in the jaws etc. of plateaued rats 5 days after transplanting three slices of placental tissue under the muscle fascia, and obtained STH-like substances from human placentae of middle stage of pregnancy. The author tried extraction of STH from the human placentae

according to the methods of isolating crystalline of STH from the pituitary gland[7]. Upon comparison of acidic and alkaline methods of isolation, it seems that the latter process is more effective in obtaining STH of stable activity[14]. NaOH,[16] $Ba(OH)_2$[15], NH_4OH[17] and $Ca(OH)_2$[7][18] were all found useful, but $Ca(OH)_2$ solution was most frequently used in this study for alkaline extraction, at pH 11.5. When the extracts thus obtained were fractionated after addition of neutral salt, STH was obtained as precipitate in globulin fractions. For example, when ammonium sulfate was used, STH precipitates at the ammonium sulfate concentration between 0.2 and 0.5 saturation. The precipitate thus obtained contained not only STH, but also other hypophyseotropic hormones and ineffective proteins, so that the extracted specimen must be processed for purifing STH protein.

Figures 3 and 4 outline graphically the changes in the level of secretion of STH-like substance and other hormones during pregnancy.

The STH-like substance is in higher concentration in the placentae in the early stage, quite like that of chorionic gonadotropin. Also like chorionic gonadotropin STH seems to be excreted from the Langhans' cells, rather than from the syncytium. Now, it has been generally accepted that the principal cells or the α-cells, which are taken to represent prolactin- and STH-secreting cells, in the anterior pituitary lobe proliferate in pregnancy, though Urasov[19] has once reported that he discovered the β-cells increased in pregnant mice. The STH-like substance in the plasma, discovered in my experiments, may be also a secretum from such eosinophilic cells.

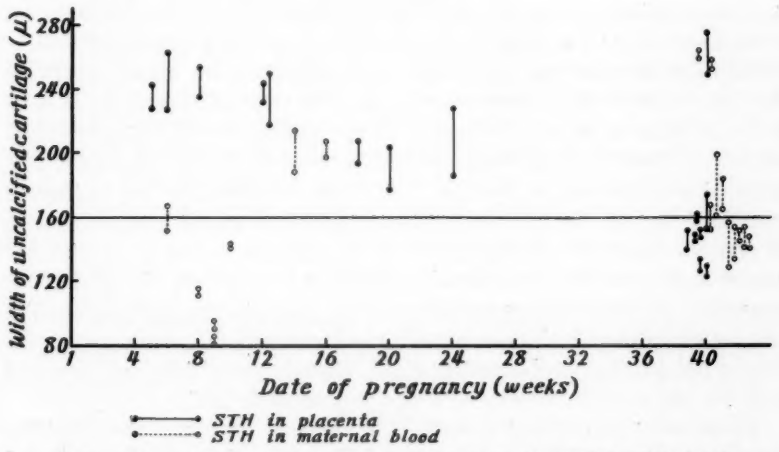


Fig. 3 The changes in the level of concentration of STH-like substance in the placenta and plasma during pregnancy.

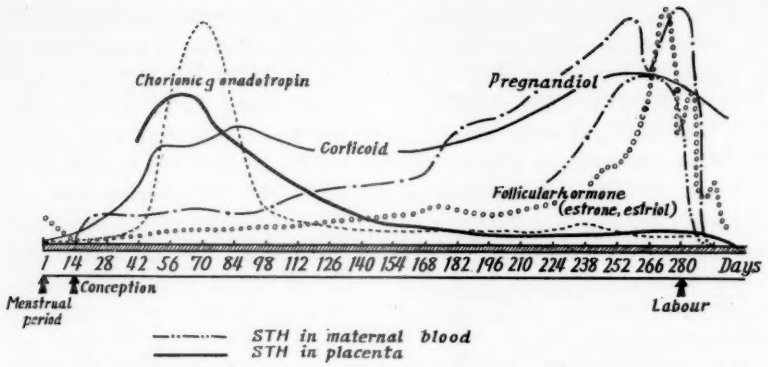


Fig. 4 The relationship between the excretion of somatotropic hormone and other hormones (chorionic gonadotropin, pregnandiol, corticoid and estrogen) during pregnancy.

From the embryological angle, it is known that the pituitary gland begins with the formation of Rathke's sac in the germinal stage, its anterior lobe is perfected by the time the fetus grows to 28 mm, and the eosinophilic cells begin to appear distinctly in the middle to the latter stage of fetal life. Smith and Dortzbach[20] found that the pituitary gland sampled from swine fetus more than 9 cm in length has the effect of stimulating growth of hypophysectomized rats. This proves that such a fetal pituitary gland already contains the so-called STH. It is, however, not thereby proved that STH acts a part in the growth of the fetus, many authors being inclined to ascribe importance to hereditary factors in the drama of fetal growth. Any hypothesis postulating actual influence of STH already demonstrated in the fetal pituitary gland on the metabolism in the fetus, or a backflow of it through the placenta into the maternal blood to be of service in the progress of pregnancy is as yet nothing but an imaginary supposition, for there is no necessary correlation between the morphological completion of an organ and its function. Moreover, we must face the problem of permeability through the placenta. The permeability of steroid hormone through the placenta is already acknowledged and the placenta is said to permit some proteins to pass through preferentially. But the few authors who studied the permeability of pituitary hormones through the placenta are mostly inclined to deny permeability of both gonadotropin and thyrotropic hormone[21][22][23]. The author could detect a small quantity of STH-like substance in the umbilical cord blood but not at all in urine of newborns and amniotic fluid.

In a non-gravid young patient with diabetes mellitus, the STH-like substance in the plasma was found increased. Forsman and Gemzell have reported on similar findings, in 24 of 40 diabetic patients, especially frequently among

juvenile cases (13/17 cases), but among them 2 showed rather retarded growth[24]. These findings seem to suggest that the delayed growth often observed in juvenile diabetic patients, is caused not by the diminished STH secretion from the hypophysis but by deficient insulin secretion. In such stunted diabetes cases, it is characteristic that the insulin deficiency is severe and that insulin tolerance is generally high in them. There is a negative correlation between the STH value and the insulin production level.

In pregnancy, several hormones from the placenta begin to be active, but their physiological significance still remains unanswered. Perhaps, it may be inferred that effect of STH lies not only in promoting growth but also in acting in mutual correlation with the other protein hormones and steroid hormones, as a biological activating hormone. Further research is needed.

CONCLUSION

Extraction of STH-like substance from human placentae of the early, the middle and the later stages of pregnancy, the blood plasma of pregnant and postpuerperal women, the umbilical cord blood, amniotic fluid and urine of newborns as well as from the plasma of a female dwarf with diabetic mellitus was undertaken, and the following results were obtained:

(1) The STH-like substance extracted from human placentae sampled in the early stage of pregnancy showed high efficacy in broadening the width of the epiphyseal cartilage, but in the later stage placentae the hormone could not broaden the zone over 160 μ, the normal average.

(2) In the maternal plasma, the STH-like substance value was high just before delivery, but rather rapidly fell off after it.

(3) A very small quantity of STH-like substance could be detected from the umbilical cord, but not at all from the amniotic fluid or the urine of newborns.

(4) There is a common tendency in the changes of concentration between chorionic gonadotropin and placental STH-like substance and, in the last stage of pregnancy, between steroid hormones and STH-like substance in the plasma.

(5) No STH-like hormone could be detected in the plasma of normal nongravidae, but it was found in a severe juvenile diabetic patient with retarded growth.

Acknowledgments

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Electrical Properties of Spider Leg Muscle

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INTRODUCTION

For the comparative physiology of voluntary muscles, it would be important to study the electrophysiological properties of arachnid muscles and the mode of their neuromuscular transmission. Researches on the coupling mechanism at neuromuscular junctions in Arthropoda have been done mainly on Crustacea[1)2)], Insecta[3)4)], and Xiphosura[5)], but on Arachnida there has been no report available. The fibers of arthropod muscles are said to be all multinucleated and probably all striated[6)]. They show histological features much different from those of the vertebrate skeletal muscle fiber. About the excitation-contraction coupling of crustacean muscle, it was suggested that certain chemical transmitter acts directly on the coupling mechanism[7)], in other words, it may be said that no propagated contraction of muscle can be evoked by an electric current. Some terestrial arthropods — spiders and insects — have the body fluid of osmotic pressure much higher than those of any other animals[8)]. Their ionic compositions are also much different from those in the vertebrate. It seems relevant to study the behavior of the membrane of the muscle adapted to such a special internal medium.

Spider leg muscles have another peculiarity. In 1906, Petrunkevicz presented anatomical evidence that all spider leg muscles are synergists, i.e., no antagonists being found. The extensions of spider legs are performed by the sudden increase in the hydrostatic pressure of the body fluid, while the flexions are carried out by flexor muscles[9)].

The present paper deals with the electrical properties of spider muscle cells which were investigated with intracellular recordings of resting, action, and electrotonic potentials.

MATERIAL AND METHOD

Experiments were carried out mainly on M. flexor patellae longus and M. flexor patellae bilobatus of Atypus karschi Doenitz (Atypidae, Prototheleae).

秩父志行

In some experiments, Metathelae species, such as Araneus ventricosus (L. KOCH), Neoscona scylla (KARSCH), N. nautica (L. KOCH) and Argiope brunnichii (SCOPOLI) were also used, but not extensively.

These two muscles were both finely striated in hematoxylin-eosin stained preparation as shown in Fig. 1. In fresh preparation, muscle fibers appear pale and opalescent. Striations were clearer in M. flexor patellae bilobatus (Fig. 1. right) than in M. flexor patellae longus (Fig. 1. left). Fiber diameters of M. flexor patellae longus ranged from 30 μ to 50 μ with the mean value of 41.9 μ. Those of M. flexor patellae bilobatus were slightly thicker and ranged from 38 μ to 58 μ with the mean of 43.7 μ.

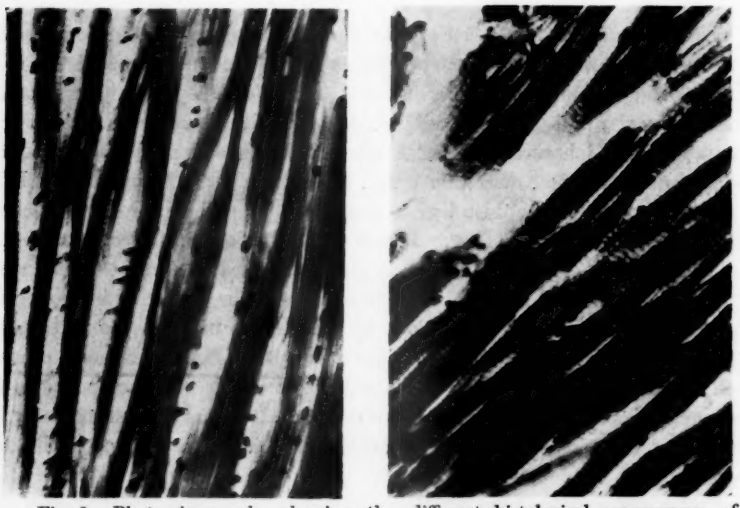

Fig. 1. Photomicrographs showing the different histological appearances of muscle fibers in M. flexor patellae longus (left) and M. flexor patellae bilobatus (right). Photographs were made from one and the same preparation. Hematoxylin-eosin staining. Magnification $\times 250$.

Legs were amputated at the coxa and fixed in a stretched position within a resin box filled with physiological saline. Muscles were exposed at the femur by cutting away the chitinous integument under visual control. Nerve bundles were dissected and split at the trochanto-femoral joint or trochanter.

As no information about the ionic compositions of the spider body fluid or the physiological saline for spiders was available, the solution of the following ionic composition was used in the present experiments, namely NaCl 8.0 g, KCl 0.1 g, $NaHCO_3$ 0.2 g, $CaCl_2$ 0.1 g, NaH_2PO_4 0.01 g and H_2O 1000 cc (pH 7.6). The ionic composition of this solution resembles that of the locust saline solution.

In fact, the solution proved to be useful to maintain the preparation in good conditions as long as four hours.

For both recording and polarization, 3M KCl-filled glass micropipettes of Ling-Gerard type, having the resistance of 10–25 meg-ohms and the tip diameter of less than 1 μ were used. For polarization, electrodes of lower resistance (10 meg-ohms or so) were selected. Potential changes were recorded with a conventional cathode-ray oscilloscope with a D. C. amplifier. The stray capacity between electrode and ground was compensated by a negative-capacity preamplifier[10] in order to measure correctly the time constant of the membrane.

The experiments were performed at room temperature of 10–20°C.

RESULTS

Resting membrane potential

The membrane potential of a muscle fiber was measured as usual with a single microelectrode, and values ranging from –46 mV to –75 mV, inside negative, were obtained. The mean value of 21 experiments was –66 mV. There was no remarkable difference in the magnitude of the resting membrane potential between both kinds of muscle. The magnitude of the resting potential decreased with the increase of the potassium ion concentration in the saline bath. Fig. 2 illustrates the relationship between external potassium concentrations and the magnitudes of the resting membrane potential. When the potassium ion concentration was decreased after application of a potassium-rich solution, reversibility of the resting membrane potential was not complete.

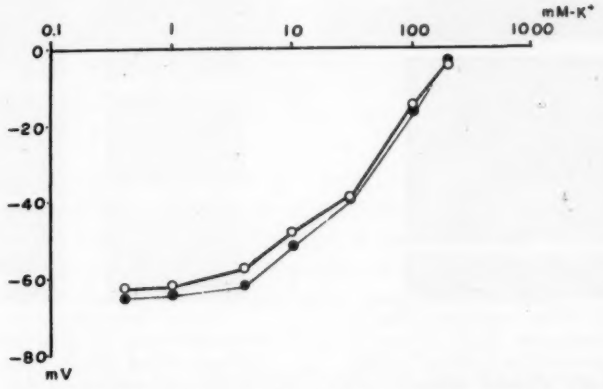

Fig. 2. Relation between resting membrane potential of two muscle fibers and potassium ion concentration in medium. Different symbols refer to different fibers.

Action potentials to nerve stimulation

When an electric pulse of 0.1 msec. in duration was applied to a nerve strand split from the nerve bundle, two kinds of action potentials, 'fast' and 'slow', were observed with intracellular recording. In about one half of all fibers, stimulation of the nerve produced a graded response, a slow depolarization of small amplitude, as illustrated in Fig. 3. The depolarization reached its summit within 2.5–4 msec. and then decayed in a more prolonged time-course. The time constant of the exponential decay was about 7.8–11.0 msec. In Fig. 3, the intensity of the stimulus was increased from bottom to top record, It can be seen that the amplitude augmented with the increase in intensity, while the time- course remained almost the same. Responses of this type did not show any sign of after-hyperpolarization. This type of response is similar in many respects to the

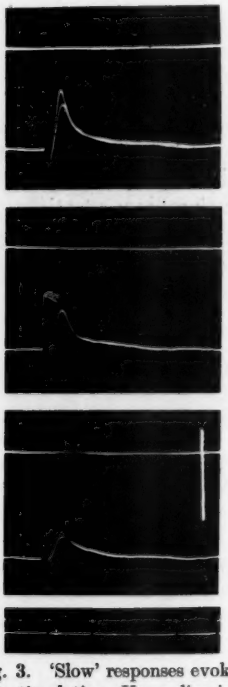

Fig. 3. 'Slow' responses evoked by indirect stimulation. Upper line in each record is potential level referred to bathing solution. Stimulus intensity was increased from bottom to top. Voltage scale as indicated by a vertical bar is 50 mV. Time mark : 10 msec.

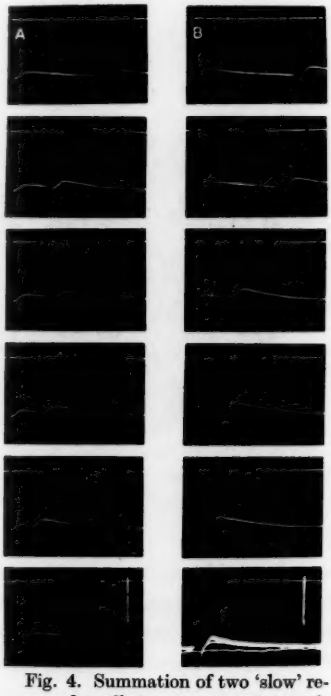

Fig. 4. Summation of two 'slow' responses of small size at various intervals between paired stimuli. Voltage scale : 50 mV. Time mark : 10 msec.

responses of the so-called slow systems which were investigated in both vertebrate and invertebrate muscles[11),12)]. Fig. 4 illustrates the summation of these 'slow' responses evoked by two successive stimuli at various intervals. The summated potential was very similar in shape to the response evoked by a stronger single shock.

The other type of response is shown in Fig. 5. This response apparently resembles the 'fast' response of insect muscle[11)12)]. This response follows the all-or-none principle. Overshoots were observed only in several cases, and never exceeded 10 mV. A step observed on the rising phase of the action potential of this type, which may be referred to a junctional potential, ranged from 15 to 32 mV in height with the mean value of 22 mV. Any special relation was not seen between the magnitude of the step and the amplitude of the action potential. Concerning the relation between the step and the resting potential, it may be said that the step height increases in proportion to the membrane potential. Production of the action potential of this second type was associated with a twitch of the muscle fiber under microscopic observation.

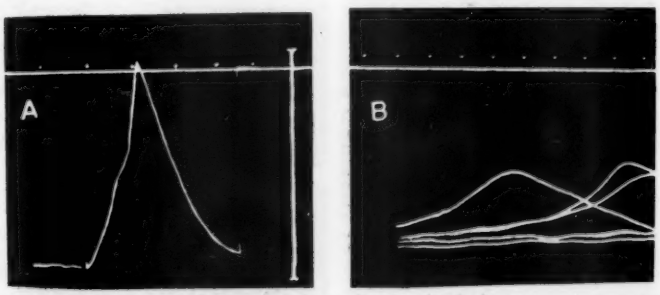

Fig. 5. 'Fast' response evoked by indirect stimulation (A) and superposed records of a train of spontaneous discharges (B). Note a step on rising phase of action potential in A. Voltage scale, 50 mV, applies to both A and B. Time mark : 10 msec.

Fig. 5 B is a superposed record of a train of spontaneous discharges which might be ascribed to some injury of the cell under test. In this record, the amplitude of the action potentials decreased in association with the decrease in step height. It is to be noted that after cessation of the spontaneous discharges, the resting membrane potential showed some considerable decrease.

Direct stimulation of the muscle fiber

When a pair of glass micropipettes, one for potential recording and the other for polarization, was impaled into a cell, the membrane potential was found sometimes smaller than in the case in which only one microelectrode was inserted into the cell. This reduction in membrane potential was caused by the second,

polarizing electrode of lower resistance; its tip diameter was probably too large for the small muscle fiber. For this reason, as a criterion for maintenance of good condition of the muscle cell, the following condition was adopted: At first the recording electrode was inserted into a cell to measure the resting potential, and then the stimulating electrode was advanced into the same cell. If the membrane potential was not reduced by more than 10% of its initial value and remained constant at least for five minutes, the preparation was used for the experiment.

In response to the cathodal polarization, most fibers showed electrotonic potential changes which were either smooth in shape or superimposed with small local potentials, as exhibited in the left-hand column of Fig. 6. These local potentials augmented with the increase in the intensity of the polarizing current. In cases of anodal polarization, there occurred no local excitatory sign (see the right-hand column of Fig. 6). The local action potentials in Fig. 6 are all monophasic and simple in form, but in some cases local potentials were oscillatory as

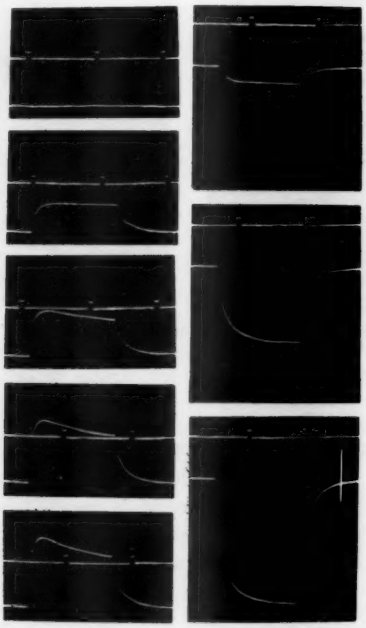

Fig. 6. Effect of direct polarization. Intensities of cathodal (left column) and anodal (right column) electrotonus were increased from top to bottom. Depolarization upwards, and hyperpolarization downwards. Local potentials can be seen only in cathodal polarization. Voltage scale: 50 mV. Time mark: 10 msec.

shown in Fig. 7. The local oscillatory potentials were recorded in about one third of the muscle fibers so far examined. The amplitude of the oscillation was small in most cases (see Fig. 7 B). Usually this local excitatory signs were observed when the cell membrane was depolarized by 20 to 30 mV. When the current intensity was high enough to generate a propagating action potential, an inflexion appeared on the electrotonic potential, and then a spike started. This inflexion may be due to a local potential. The inflexion mentioned above seemed to arise when the depolarization reached 12 to 22 mV. The threshold for the spike ranged from 16 to 28 mV, and the current intensity needed to reach the threshold ranged from 0.28 to 0.44 μ A. The over-all amplitude of the spike in

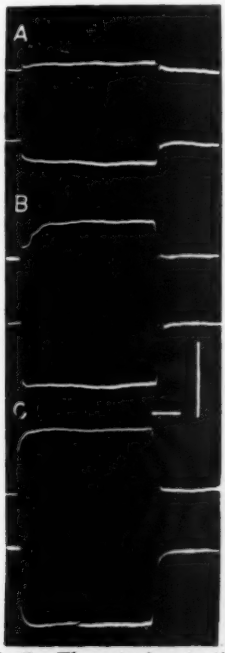

Fig. 7. Electrotonic potentials with local oscillatory potentials. As shown in each pair of records, anelectrotonic potentials are larger than catelectrotonic potentials for the same intensities of polarizing currents but in opposite directions. In B, weak oscillatory potentials are superposed on rising phase of catelectrotonic potentials. Voltage scale: 50 mV. Time mark: 10 msec.

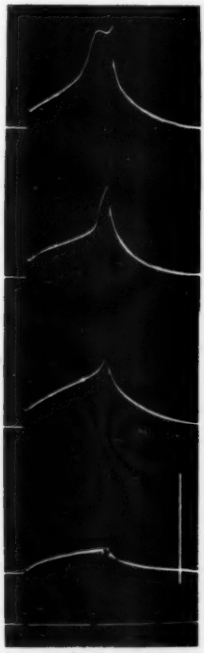

Fig. 8. Directly evoked propagating action potential. Current intensities were increased from bottom to top. Voltage scale: 50 mV. Time mark: 10 msec.

response to direct polarization ranged from 48 to 72 mV. Such a propagating action potential was always accompanied by a rapid contraction of the muscle fiber.

It is easy to obtain propagating action potentials by indirect stimulation, but far more difficult by direct stimulation; in the present experiments propagating action potentials were observed only in one case of M. flexor patellae longus and four cases of M. flexor patellae bilobatus.

Electric constants of the muscle fiber

The so-called 'rectangular pulse technique'[13),14)] was applied to the muscle fibers of both kinds to analyse the cable properties of the cell membrane. The measurements were done on the effective resistance and the time constant of the membrane. The diameter of the fiber on which the electrical measurements were performed was gauged with a microscope micrometer.

With the values of the effective resistance, the time constant and the diameter of the fiber, and with an assumed value for the specific longitudinal resistance of the fiber, the other constants, such as the specific membrane resistance, the membrane capacity, etc., were calculated from the following equation, which holds for the subthreshold electrotonic potentials.

$$\frac{V}{I} = \frac{1}{2}\sqrt{r_m \cdot r_i} \cdot \exp\left[\frac{-x}{\sqrt{r_m/r_i}}\right] \quad \cdots\cdots (1)$$

where V denotes the electrotonic potential caused by a steady current, I, r_m the transverse resistance of the membrane times unit length, r_i the longitudinal resistance of the fiber per unit length, and x the distance between polarizing and recording electrodes. Provided that x becomes very small, the equation will be simplified as follows:

$$R_e = \frac{V}{I} = \frac{1}{2}\sqrt{r_m \cdot r_i} \quad \cdots\cdots (2)$$

where R_e is the effective resistance of the membrane.

r_i may be calculated by the next formula,

$$R_i = \pi \cdot \rho^2 \cdot r_i \quad \cdots\cdots (3)$$

from the measured radius of the fiber, ρ, and the assumed specific fiber resistance, R_i.

Hence the value of r_m can be obtained from the relation (2).

Then the specific membrane resistance, R_m, will be obtained from the next relation,

$$R_m = 2\pi \cdot \rho \cdot r_m \quad \cdots\cdots (4)$$

The length constant or the space constant of the fiber, λ, may be derived from the relation

$$\lambda = \sqrt{\frac{r_m}{r_i}} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (5)$$

Knowing the time constant, τ_m, from the time needed to rise to 85% or to decline to 15% of the electrotonic petential at $x=0$, the specific membrane capacity, C_m, is calculated as follows:

$$C_m = \frac{\tau_m}{R_m} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (6)$$

In the following experiments, two microelectrodes were inserted into the same cell as closely as possible to each other. In reality the distance between them was not longer than 25 μ. This value is practically very small in comparison with the length constant of the fiber. Under such conditions the formula (2) may be used. When I had been in a position to record potential curves with two separate electrodes inserted into a single muscle cell, the value of the longitudinal resistance R_i would have been determined experimentally. In reality, however, the muscle cell was too fragile and too small in diameter, to allow the insertions of both recording microelectrodes at two separate points. Therefore the value of R_i had to be assumed. In assuming the value of R_i the following facts were taken into consideration. It is known that the ratio of the specific longitudinal resistance R_i to the specific resistance of the medium R_0 is not much different

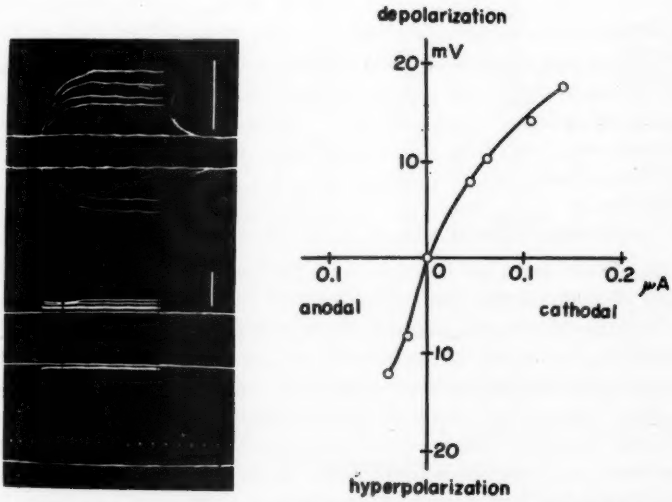

Fig. 9. Electrotonic potentials (left upper) caused by long polarizing currents (left lower) and current-voltage relationship (right). Voltage scale: 50 mV. Current scale: 0.2 μA. Time mark: 10 msec.

from species to species and from tissue to tissue of the same animal. According to Yamasaki & Narahashi[15], the mean value of R_i/R_0 ratio for several kinds of excitable tissues is 2.72. The osmotic pressures of the body fluid corresponds to 262.3 mM NaCl solution, because the depression of the freezing point of the fluid is reported as –0.894[8]. The specific resistance of 262.3 mM NaCl solution was calculated to be 51 ohm cm at 12°C. Therefore a value of 140 ohm cm may be obtained for R_i.

R_e of the fiber was determined from the slope of the current-voltage relationship as illustrated in Fig. 9. The relation is linear in a restricted range of the current; towards higher values of current the rate of increase in voltage becomes smaller against that in current, so that the slope resistance determined over higher values of current is lower.

Results obtained from seven experiments are summarized in Table I. Generally speaking, the membrane capacity of the spider's muscle fiber is large, and the specific membrane resistance thereof small compared to those of the vertebrate twitch fibers[14),16),17]. C_m and R_m of spider muscle fibers were similar to those found in marine crustacean muscle fiber[18),19]).

TABLE I. Membrane Constants of Spider Muscle Fibers

	No. of preparation	ρ $\times 10^{-4}$cm	R_e $\times 10^3\Omega$	τ_m $\times 10^{-3}$sec.	R_m Ωcm^2	C_m μF/cm^2	λ $\times 10^{-1}$cm
M. flexor patellae longus	1	25	150	11.4	197.8	57.6	0.420
	2	20	142	5.2	90.9	57.2	0.254
	3	20	168	6.2	127.2	47.2	0.301
	4	20	157	8.9	111.0	80.2	0.280
	mean	21.3	154.8	7.9	131.7	60.6	0.314
M. flexor patellae bilobatus	5	25	205	12.5	370.2	33.7	1.82
	6	25	277	18.3	675.0	27.1	2.46
	7	25	231	12.0	467.9	25.6	2.04
	mean	25	234.3	14.3	504.4	28.8	2.11

The membrane capacity is larger, and the membrane resistance smaller for the fibers of M. flexor patellae longus than for those of M. flexor patellae bilobatus. In other six experiments (not shown in Table I) the difference in R_m and C_m between the two kinds of muscle was seen as clearly as in Table I, but in further two cases distinction was not so clear, the values of R_m and C_m being intermediate between the distinctly different two groups mentioned above.

DISCUSSION

The magnitude of the resting membrane potential of spider muscle cells is nearly of the same order as that found in marine crustacean muscle fibers[18)], and is larger than that found in muscles of Limulus polyphemus Latr.[5)]. The

diameter of fibers is 180 μ on the average in the Crustacea, and 25–40 μ in the horseshoe crab. As stated above, the diameter of the spider muscle fiber ranged from 30 μ to 50μ. Therefore it can be said that the spider muscle fiber is akin to the marine crustacean muscle fibers concerning the membrane potential, but to the Limulus muscle fiber concerning the fiber size.

It is said that some fibers are doubly innervated so that they show 'slow' and 'fast' responses depending upon the kinds of nerve stimulated. In the present experiments one and the same microelectrode inserted into a muscle cell was found sometimes to record a 'slow' response to stimulation of one nerve strand, but a 'fast' one to stimulation of another. 'Fast' and 'slow' responses were obtained in 39 cases, 'fast' ones only in 33 cases, 'slow' ones only on 4 cases and none in 18 cases. The 'fast' response illustrated in Fig. 5 A is very similar to 'fast' action potentials in crustacean and insect muscles. The percentage of spider muscle fibers which produced slow responses only was small in comparison with those of insect muscle fibers.[20] 'Slow' responses increase with the stimulus intensity, but never exceed about 35 mV, and are always smaller than 'fast' responses. A step on the rising phase of the 'fast' response is not constant in its magnitude and the clearness in appearance. The step probably indicates a junctional potential. In view of the fact that the step appeared distinctly in most cases it is likely that the neuromuscular junction of the spider muscle is not diffuse in character, but localized to some extent, although no histological examination has yet been carried out.

In most cases of direct stimulation, weak local oscillatory potentials appeared superposed on the electrotonic voltage shift, but exceptionally large propagating action potentials could be observed (Fig. 8). Even when the direct depolarization was over the magnitude of the junctional potential, no propagating action potential appeared in most cases, only oscillatory ones prevailing. In short, it is more difficult to evoke propagating action potentials by direct stimulation in spider muscle fibers than in vertebrate ones. The magnitude of oscillatory potentials was generally much smaller in spider muscle fibers, than in crustacean and insect muscle fibers.

The membrane capacities of spider muscle fibers are surprisingly large in comparison with those of vertebrate muscle fibers. The question as to whether this difference in membrane capacity depends on any structural difference of membranes can not be answered at present, for detailed histological and electron-microscopical data are lacking.

It is interesting to find differences in many respects between both kinds of spider muscles. The fibers of M. flexor patellae bilobatus are characterized by small membrane capacity, large membrane resistance, and high responsiveness with propagating action potentials to direct stimulation. In this sense, the fibers of M. flexor patellae bilobatus may be said to be more akin to vertebrate

muscle fibers.

SUMMARY

Electrical properties of the spider muscle fiber were examined on M. flexor patellae longus and M. flexor patellae bilobatus of Atypus karschi DOENITZ.

1. The resting membrane potential ranged from –46 to –75 mV (mean –66 mV).
2. By stimulation of nerve strands, two kinds of responses, 'fast' and 'slow', were intracellularly recorded from a single muscle fiber.
3. 'Fast' responses consisted of initial slow and later abrupt rises of potential. The former is probably a junctional potential and the latter a spike having the all-or-none character.
4. 'Slow' responses are of graded and summative nature.
5. Most fibers produced only electrotonic and local potentials in response to direct catholdal polarization, but propagating action potentials were observed in a few cases.
6. The local potencials were oscillatory in most cases. The amplitudes of oscillations were, however, small compared to those in crustacean muscle fibers.
7. Electric constants of the fiber membrane were determined as follows: The effective resistance, R_e, was 154.8 KΩ on the average; time constant, τ_m, 7.9 msec.; specific membrane resistance, R_m, 131.7 Ωcm^2; membrane capactiy, C_m, 60.6 μF/cm^2; and length constant, λ, 0.314 mm in M. flexor patellae longus. The corresponding values obtained from M. flexor patellae bilobatus were as follows; R_e 234.3 KΩ; τ_m 14.3 msce.; R_m 504.4Ωcm^2; C_m 28.9 μF/cm^2; and λ 2.11 mm. In these calculations the specific longitudinal resistance, R_i, was assumed to be 140 Ωcm.
8. The data were discussed from a comparative physiological point of view.

I wish to express my gratitude to Prof. K. Motokawa for his invaluable advice in the experiments and in preparing manuscripts.

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Epidemiological Studies on Hypertension and Cerebral Haemorrhage in North-east Japan

By

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Vascular lesions affecting the central nervous system are now the most important cause of deaths in Japan and are particularly common in the rural districts of the north-east of the main island where the blood pressure level of inhabitants is known to be higher than in other areas. Figure 1a (male) and 1b (female) show the geographic distribution of pre-senile (45–64 years) death rates from cerebral haemorrhage for 1955. [The term "cerebral haemorrhage" will be used in place of "Vascular lesions affecting central nervous system (B22)" because it is by far the most common cause of death in the senile and pre-senile period.] The distribution of deaths from cerebral haemorrhage has been discussed in an earlier paper which was mainly concerned with data from the north-west part of this area[1].

In the present paper the relationship between hypertension and various environmental factors – physical, nutritional, social and economic – is examined in data collected at a later date from the Pacific side of the north-east. Blood pressure was measured in the population of a number of farm and fishing villages and among employees of a chemical works and a coalmine. Blood pressure was taken by the authors, using the mercury sphygmomanometer, with the person in the supine position after several minutes rest in that position. At the same time living conditions and other environmental circumstances were investigated.

I. Sources of Data

The localities of the following villages, factory and coalmine are shown in Figure 2.

1. *Farming village A* (Ohira-mura) is in a single crop area (rice) in the

高橋 英次, 加藤 勝雄, 川上 吉昭, 石黒 弘三, 金田 昭治, 小林 悟, 大庭 英子
矢野 薫夫, 伊藤 裕一, 白石 真, 村上 直人, 菅原 恒有, 目黒 勇平, 鈴木陽太郎

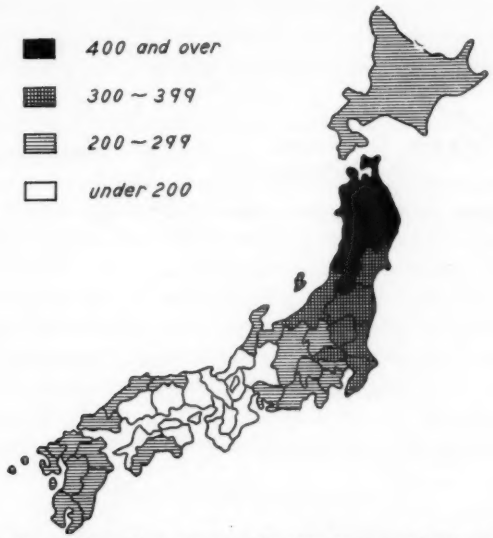

Fig. 1a. Distribution of pre-senile (45~64 years) death rate from cerebral haemorrhage by prefectures in Japan (1955, male).

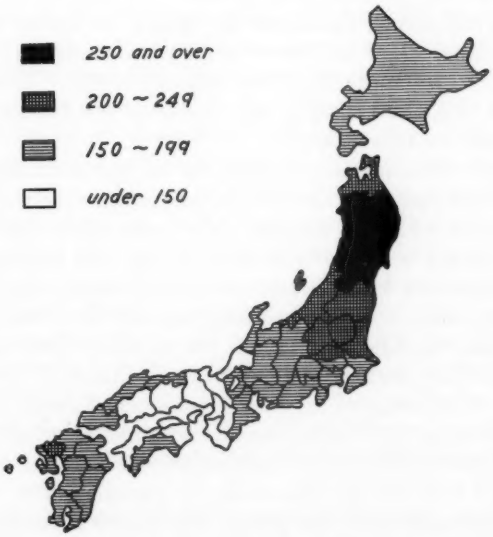

Fig. 1b. Distribution of pre-senile (45~64 years) death rate from cerebral haemorrhage by prefectures in Japan (1955, female).

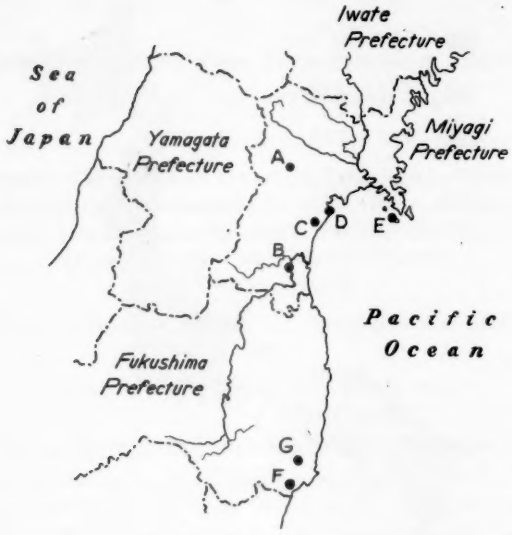

Fig. 2. Location of areas studied.

middle-west part of Miyagi Prefecture and has a population of 6,454 (1,030 families). The crude death rate from cerebral haemorrhage per ten thousand population was 14.7 in 1950, 15.2 in 1955 and 23.0 in 1956. Blood pressure was measured on 85–97% of all villagers over 5 years of age four times a year (seasonally) from 1957 to 1958 inclusive.

2. *Farming village B* (Kitagô-mura) lies on the river Abukuma in the southern part of Miyagi Prefecture and is also a single crop farm village. The population is 5,058 with 778 families. The crude death rate from cerebral haemorrhage was 13.3 in 1954, 16.9 in 1955, 15.5 in 1956, 10.0 in 1957 and 11.9 in 1958. Blood pressure was measured on 91% of villagers over 19 years of age.

3. *Farming village C* (Gamoh, Takasago-mura) lies in the administrative district of Sendai. Though the village is on the Pacific coast, the inhabitants do little fishing. They are single crop cultivators. About 30 families were selected at random to investigate diet, blood chemistry and blood pressure.

4. *Fishing village D* (Shichigahama-mura) is north of farm village C and is divided into three settlements. The northern one faces the beautiful bay of Matsushima, and most of the inhabitants are concerned with the shallow-sea cultivation of oysters and laver (sea weed). The eastern settlement directly faces the Pacific Ocean, and the inhabitants are deep-sea fishermen particularly in the Northern Pacific. In the southern settlement the principal occupation of the

inhabitants is farming, but they also do a little coast fishing. The population is 13,118, with 1970 families. Blood pressure was measured on 90% of villagers aged 20 years and over, for 3 seasons (summer, autumn and winter) during 1958 and 1959.

5. *Fishing village E* (Aji-shima) with a population of 3,144 (477 families) is on a small island near the south coast of the Oshika peninsula. The island is relatively flat, and in the fields barley, wheat and soya beans are planted, but very little rice is grown. The inhabitants engage in both coast and deep-sea fishing. The crude death rate from cerebral haemorrhage was 6.4 in 1949–1953 and 38.2 in 1954–1958. As in farm village C, about 30 families were selected at random to investigate diet, blood chemistry and blood pressure.

6. *Chemical factory F* is in a city in the south east of Fukushima Prefecture. Most of the labourers are natives of the province. Over 90% of all employees (1,196 males and 249 females aged 15–54) were studied.

7. *Coal mine G* is near chemical factory F, in the Fukushima Prefecture, and 1,097 underground and 447 surface workers aged 20–54 years were studied.

II. Heredity and Environment

There have been many familial studies of hypertension and there can be little doubt that there is a substantial genetic component in its aetiology.

Ohba[2] classified school children and their parents in farming village A into high, medium and low blood pressure groups and found that the blood pressures of children and of their parents were highly correlated ($P<10^{-10}$). It should be remembered, however, that this village is in the north-east area where blood pressure is known to be higher than in other parts of Japan.

However, an hereditary explanation of the high incidence of hypertension in the north-east area is open to serious criticism. Suzuki[3] also classified families in a farming village near village A into high, medium and low blood-pressure groups. He collected data on the blood pressure of 174 emigrants from this village to other parts of the country, (most of these emigrants had left their homes before the age of 20), and could not find any correlation with pressures in other members of families to which they belonged.

Itahara[4] investigated the second generation (20–40 years of age) of farm immigrants in Hokkaido from Akita Prefecture, which is situated in the north-esat and has the highest death rate from cerebral haemorrhage in Japan. Their blood pressure was rather lower than that of farm-villagers in Akita Prefecture, and did not differ much from that of other villagers in the same district of Hokkaido.

The north-east is a less well-developed part of Japan, and the rural inhabitants still follow the traditional way of life, particularly as regards their diet and dwellings. It is possible therefore that environmental rather than genetic

factors may determine the familial incidence of hypertension.

III. Effects of the Physical Environment

A. Temperature

Sugawara[5], using data from Iwate Prefecture, has demonstrated a seasonal periodicity of deaths from cerebral haemorrhage by means of correlogram through calculation of autocorrelation coefficient[6]. He also showed that temperature is a more important factor than barometric pressure in the seasonal variation of mortality from cerebral haemorrhage. Takahashi *et al.*[1] had previously attempted to explain the geographical variation (mortality is lower in the south-west of Japan and higher in the north-east, except in Hokkaido) in terms of temperature. In the main island and in the southern islands where rural inhabitants have no heating in their houses during winter, atmospheric temperature appears to be an important factor. But in Hokkaido, where stoves are used for heating the houses, the death rate is not so high as in the north-east area in the main island, notwithstanding that the climate is more severe, especially in winter. Blood pressure levels and the prevalence of hypertension in the adult of presenile population seem always to be closely related to death rate from cerebral haemorrhage.

From the sources mentioned above the effect of environmental temperature

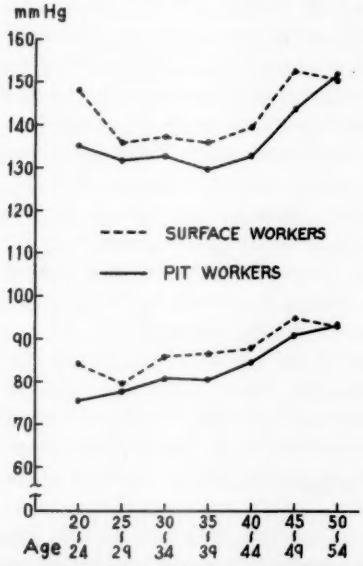

Fig. 3. Comparison of blood pressure levels between surface and underground workers in coalmine G.

upon blood pressure level was studied. The results were as follows:

1. For each population group mean blood pressure level was always higher in winter than in summer.

2. In mines of coalmine G both temperature (30–40°C) and humidity (over 95%) are always high and sometimes the face workers suffer from heatstroke. Murakami[7] compared blood pressure levels, diet and living conditions of 1097 underground and 447 surface workers. No significant difference of diet or living conditions was found between these two groups. (Diet was surveyed at random, 20 families in each group, using the methods of the Japanese National Nutritional Survey.) As shown in Figure 3, for each 5-year age group (except 50–54) both systolic and diastolic blood pressures were lower for underground workers. Although the distribution of blood pressure, especially in higher age classes, is not strictly normal, probability products[8] have been calculated for all

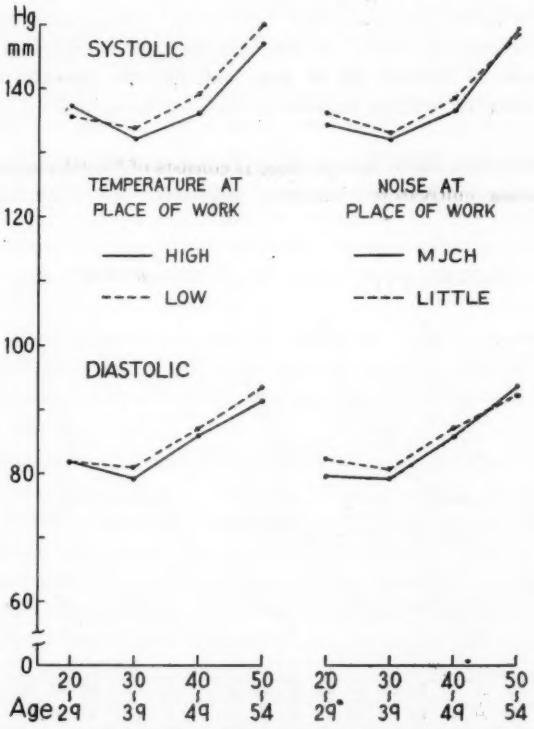

Fig. 4. Comparison of blood pressure levels between workers of hot and not-hot post, and noisy (over 95 phon) and not-noisy post.

age classes. There were significant differences between underground and surface workers for both systolic and diastolic blood pressure levels ($P<0.005$). The same was true for the incidence of hypertension. Although there were some differences between the groups in incidence of hypertension in families, annual income and hours of sleep, the only factor of importance appeared to be temperature at place of work.

Blood pressure levels of coalminers reported by Murakami[7] seem generally higher than those reported by Akaike[9] and Watanabe *et al.*[10] which were measured just after the war in the coalmines of the same district. This may be due to dietetic changes after the war.

3. In a steel foundry K, in Iwate Prefecture, certain employees are exposed to excessively hot working conditions. As shown in Figure 4, the mean blood pressure of such workers is lower than that of workers in cooler surroundings for each 5-yearly age group (except 20–24, in which the numbers were small). Probability products of the difference between the two groups through all age classes are significant ($P<0.01$). In the same way, the incidence of hypertension (systolic pressure of 150 mm Hg or over, and diastolic pressure of 90 mm Hg or over) was lower for workers exposed to excessive heat than for those in cooler occupations.

4. As mentioned above fishing village D consists of 3 settlements of different types: shallow-sea cultivators; deep-sea fishermen; and coast-fishing farmers.

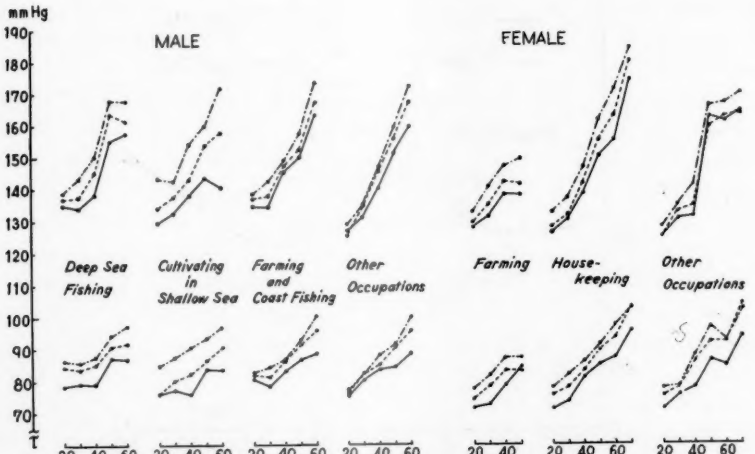

Fig. 5. Seasonal variation of blood pressure levels of villagers grouped by occupation in fishing village D.

Workers in the village can be divided into the same 3 occupational groups with the addition of "other occupations" containing retailers, service workers, officials etc. The range of seasonal variation of blood pressure level is different for the different occupational groups as seen in Figure 5[11]. In summer, both the systolic and diastolic blood pressure levels of shallow-sea fishers are the lowest of all the male occupation groups, but in winter they are among the highest. For shallow-sea fishers who cultivate oysters and laver (sea weed) winter is the busiest season. When working their hands are immersed in sea water at a temperature of 3–4°C. The air temperature is 0–2°C and there is always an air current of about 2–3 m/sec. (dry Kata-index about 36, wet Kata-index about 47).

Deep-sea fishermen on the other hand have a higher blood pressure in summer, but the rise in winter is not so great, and the seasonal variation is therefore comparatively small. They fish for salmon and trout from April to September and for codfish from October to December, usually in the north Pacific Ocean and in ships of rather less than 100 tons.

The seasonal range for shallow sea cultivators is on the average 17.4 mmHg for systolic and 11.4 mm Hg for diastolic pressure. This is the greatest range among all the male occupation groups ($P<0.01$). Seasonal variation of systolic blood pressure for deep-sea fishermen is smaller than that for farmers who are also coast fishers ($P<0.05$). The group of "other occupations", which contains more indoor workers, in general shows smaller seasonal variation for both males and females. Some public institutions in this province are heated by stoves in winter.

Although many other environmental influences may be involved in the aetiology of differences in the range of seasonal variation among different occupational groups in fishing village D, it seems likely that exposure to cold is of major importance.

5. As a rule, Japanese houses are protected more against the heat in summer than against the cold in winter. Although there is rather more sunshine in winter on the Pacific side of this area than on the Japanese Sea side, the temperature sometimes falls to –10°C at night, and heating by stove is uncommon. Possession of a ceiling in the living room or bedroom seems to some extent to regulate the indoor temperature. In farm village B and fishing village D two groups were defined according to whether or not the living rooms and bedrooms had ceilings. No significant difference of blood pressure level was found for all age groups together, but for females 40–69 years old the group without a ceiling in their living room had a higher systolic pressure than the group with a ceiling ($P<0.05$). In fishing village D also, the group without a ceiling shows a higher diastolic pressure ($P<0.01$).

Takahashi *et al.*[1] have reported previously that the effect upon blood pressure of indoor temperature through stove heating is more marked in females than in males. This seems to be due to the fact that in the cold months of the winter housewives spend more time indoors than men.

Although it is not possible to be sure about the relation between physiological rise in blood pressure brought about by environmental coldness [recently reported in detail by Brod[12]] and permanent hypertension, it seems likely that continual exposure to cold may prove to be one of the most important factors in the aetiology of hypertension.

B. Noise

It is known that blood pressure tends to rise when a person at rest is exposed to noise of more than 90 or 100 decibels. It is of interest therefore to enquire whether or not workers in noisy occupations have a tendency to hypertension.

1. We had the opportunity to measure the blood pressure of wireless workers in a central meteorological observatory where there is almost always a background noise level of 72–75 phon, and this sometimes rise to 80–85 phon when there are typhoons in the province. The mean blood pressure of 24 male workers aged 26–37 was 124.5 (range 120–129, $P<0.05$) mm Hg systolic and 69.3 (66–73)mm Hg diastolic. These levels were no higher than for 665 male teachers of the same age in this province measured by us[13], and were lower than for farming and fishing villages A, B and D.

2. In newspaper office H there is at the printing presses a background noise of about 105 phon and in the foundry of about 95 phon. The blood pressure of 18 male workers aged 18-38 was 120.7 (115–126) systolic and 70.9 (66–76) diastolic. These values are rather low, but it must be remembered that in the foundry there is a working temperature of 30°C.

3. There are also noisy occupations in steel foundry K (Kamaishi) in Iwate Prefecture. Blood pressure levels of workers in noisy occupations (always above 94 phon) were compared with those of workers in less noisy occupations. As shown in Figure 4, the pressure levels in the noisy occupations appeared to be rather lower but the differences were not striking.

It seems reasonable to conclude therefore that although noise results in a physiological rise of blood pressure, noisy working conditions do not appear to be of importance in the aetiology of hypertension.

IV. Effect of Diet

A. Present data

In the present epidemiological studies blood pressure is related to: rice consumed daily (expressed as number of rice-bowls); amount of "miso" soup (made from soya-bean paste), which is one of the most important items of diet for

farmers in the north-east of Japan and is expressed in number of soup-bowls; salt, sweet or spice preference; drinking and smoking habits etc.

1. Consumption of rice

Larger rice-bowls are used in rural than in urban districts. Males aged 20–30 years in these rural areas usually take between 10 and 12 bowls of rice daily, the amount decreasing with age. Almost 80% of villagers mix a little barley with the boiled rice.

In farm village A males aged 25–39 who ate 10 or more bowls of rice showed a higher blood pressure level than those who ate less than 10 bowls ($P<0.01$), and females aged 25–49 who ate 7 or more bowls a higher systolic than those who ate less than 7 bowls ($P<0.001$) (see Fig. 6). However, for males aged 40–69 the reverse was true, and therefore for all ages taken together the difference between big eaters and small eaters was not significant. In farm village B and fishing village D there was also no significant difference.

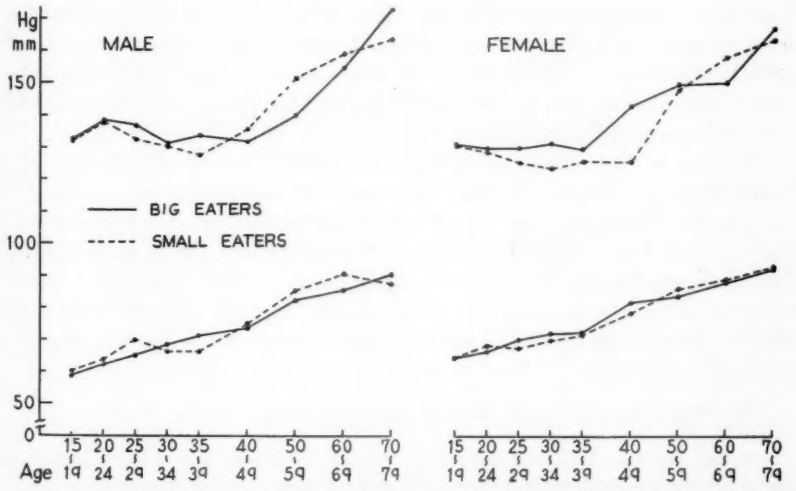

Fig. 6. Comparison of blood pressure levels according to amount of rice eaten in farm village A.

Note; Big eaters: males 10 bowls and females 7 bowls of rice or more per day.
Small eaters: males 9 bowls and females 6 bowls of rice or less per day.

However, in chemical works F, males who were big eaters (9 or more bowls of rice) showed higher blood pressure levels and a higher incidence of hypertension than smaller eaters[14]. This difference was most marked in the 15–29 age group ($P<0.01$).

In rural districts of Japan, especially in the north-east, it is a custom to eat

a great deal of glutinous rice "mochi" in the New Year (lunar calendar). It was found that the blood pressure, both systolic and diastolic, of middle school children in village A[2] was higher 2 weeks after the New Year than it was immediately before.

2. Salt intake with "miso"-soup

"Miso", the base of "miso"-soup, is made of soya beans, rice yeast and salt, and in rural districts is usually home-made. "Miso"-soup is one of the most important sources of salt for country people. The "miso"-soup consumed by farmers has in general a higher salt content than that consumed by town dwellers [Sasaki *et al.*[15]]. At the same time, whereas town dwellers usually take 2–4 bowls of "miso"-soup a day, rural inhabitants, especially in the north-east, may take up to a dozen bowls per day.

In the present investigation no great differences of blood pressure level could be found between the big and small eaters of "miso"-soup in farm village A and B (except males aged under 60 in the latter village in which the group of big eaters showed higher systolic blood pressure level than that of small eaters – $P < 0.01$). However, in fishing village D, where at every meal inhabitants take "miso"-soup in which they boil vegetables and fish, the group of big eaters showed a rather lower blood pressure level than the group of small eaters.

Workers in factory F generally take a smaller amount of "miso"-soup than farm and fishing villagers, especially those under 30 years of age who are mostly living in dormitories and take only 2 bowls a day. Workers over 30 years old, most of whom live at home, take about 3–5 bowls, a day. Among male workers aged under 40, the big eaters showed higher blood pressure levels and a higher incidence of hypertension than the smaller eaters ($P < 0.05$). However, for male workers under 40 the number of bowls of "miso"-soup taken a day is highly correlated with the number of bowls of rice taken ($P < 10^{-4}$).

3. Preference for salted pickles and salty food

Salted pickles of radish, cucumber, egg-apple, greens and other vegetables are a common side-dish for most villagers, especially in the north-east area. Sometimes the meal consists only of rice, "miso"-soup and salted pickles, but more commonly a little fish is added once or twice a day or at least once every two days. The amount of salt in the pickles is generally higher in the rural than the urban districts and the same is true of the "miso"-soup. Also it is probable that they take more salt because cereals and vegetables are deficient in Na but rich in K and they lose much Na when they sweat in their work in the open under the sun. However, excessive intake of salt seems to raise the metabolic rate and to increase tolerance to cold in winter [Ogata *et al.*[16]].

In farm village B blood pressure levels were compared for three groups, those

who liked salted pickles, those who disliked them and an intermediate group. The number of persons in the "disliked pickles" group was too small for analysis, but the group who liked pickles showed higher blood pressure levels than the intermediate group ($P<0.001$).

In addition the same inhabitants of farm village B were divided into 3 groups according to whether they were fond of sweet, pungent or salty food. The group fond of pungent food was very small. But the blood pressure level of persons fond of salty food was higher for both sexes and at all ages than that of persons fond of sweet food ($P<0.001$) (Fig. 7).

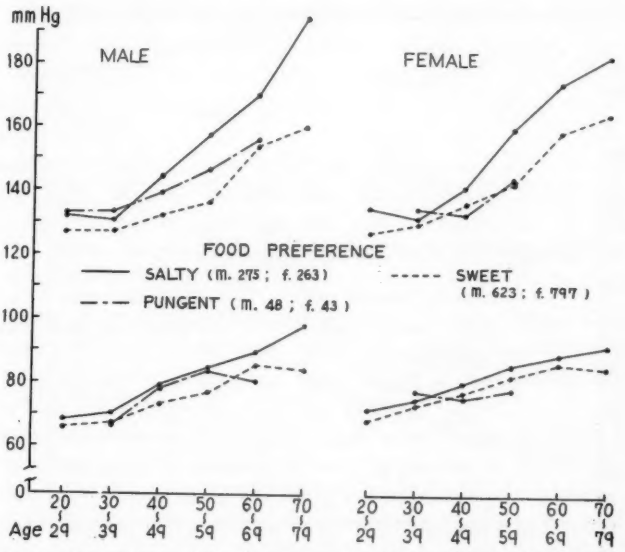

Fig. 7. Comparison of blood pressure levels according to food preference in farm-village B.

Among male workers in factory F, there was a significant difference of blood pressure level and of incidence of hypertension between sweet-fond and non-sweet-fond groups at all ages, and this was particularly marked at ages above 39 ($P<0.01$).

4. Drinking habits

The consumption of the Japanese alcoholic drink "saké", which is made of rice, in general increases with age among males in these rural districts.

In farm village A blood pressure levels were compared for 3 groups–abstainers, opportunity drinkers and habitual drinkers. Among males, the habitual drinkers

showed significantly higher systolic blood pressure levels than abstainers for all age groups ($P<0.01$), and the tendency was most marked in the age group 50–59.

In farm village B there were very few habitual drinkers, most drinkers being opportunity drinkers. When male adults were divided into 3 groups–abstainers, light drinkers (1–3 "gô" which is equal to about 180–540 ml) and heavy drinkers (over 3 "gô"), in the age group under 30 there was no appreciable difference. For older men, however, the difference was very marked (Fig. 8) and both systolic and diastolic pressures were higher for heavy drinkers than for the other 2 groups ($P<0.001$). Systolic blood pressure of abstainers seemed to be a little higher than that of light drinkers in 50–59 and over age classes ($P<0.02$).

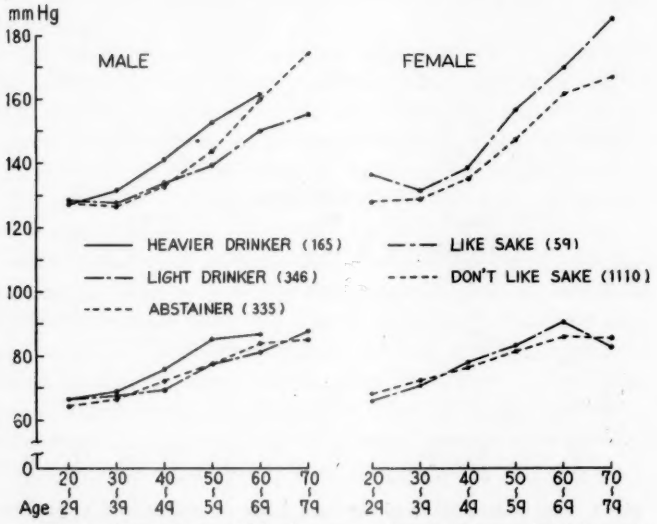

Fig. 8. Comparison of blood pressure levels between drinkers and abstainers in farm-village B.
Note; Heavier drinker: Over about 540ml of saké.

In fishing village D, almost all drinkers belonged to the habitual group, and were divided into 2 sub-groups, light drinkers (1–2 "gô") and heavy drinkers (3–4 "gô"). Systolic blood pressure of males aged 50 and over was lowest for the light drinkers, as shown in Figure 9, and the differences between them and the heavy drinkers and abstainers was significant (respectively, $P<0.001$ and $P<0.01$). It is not clear why this should be so. It is possible, however, that the higher blood pressure of abstainers in this age group (50 and over) may be due to voluntary abstention from alcohol following the onset of hypertension.

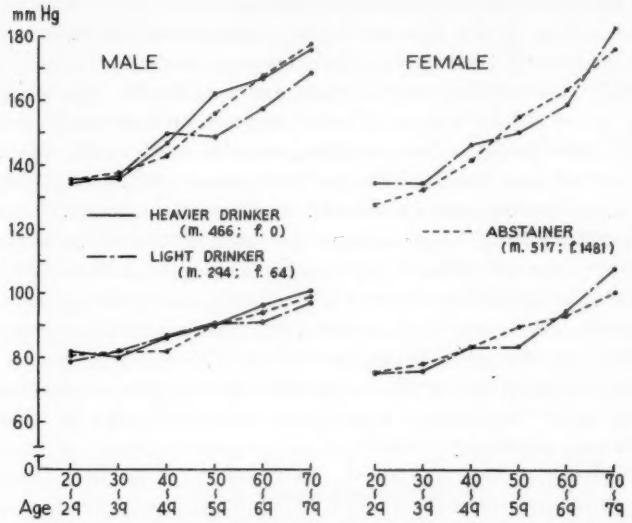

Fig. 9. Comparison of blood pressure levels between drinker and abstainers in fishing village D.

There are very few female habitual drinkers. In farm village B, the systolic blood pressure of females who liked "saké" was higher than that of those who disliked it ($P<0.01$). However, in farm village A and fishing village D no difference was found between these two groups.

Among male workers in factory F, where there is an upper age limit of 55 years, the incidence of hypertension in evening drinkers in 30–39 age group was higher than that of abstainers ($P<0.05$) and opportunity drinkers occupied an intermediate position. The blood pressure of habitual drinkers was higher than that of abstainers at both systolic ($P<0.05$) and diastolic ($P<0.01$) levels.

To summarize; habitual drinkers above age 30 or 40 showed higher blood pressure levels in farm village A and in factory F. However, when inhabitants of farm village B and fishing village D were classified by the amount of alcohol consumed it was found that light drinkers had a lower systolic pressure than abstainers or heavy drinkers.

5. Smoking habits

In farm village A non-smokers, light smokers (10 or fewer cigarettes per day or a packet of 30 gm cut tobacco per 2½ days) and heavy smokers (more than 10 cigarettes per day) were compared. Among males aged 40–69, heavy smokers had the highest and non-smokers the lowest systolic pressures ($P<0.01$). However, there was no such difference for diastolic pressure and for females no

difference for either systolic or diastolic pressure.

In farm village B male light smokers had lower systolic blood pressure levels not only than heavy smokers but also than non-smokers ($P<0.01$, $P<0.05$, respectively). For females, however, there was no difference. In fishing village D and in factory F there was no difference between the three smoking groups.

Most studies in Japan have reported negative results with regard to the effect of smoking upon blood pressure; but Shibayama[17] had noted that moderate smoking made blood pressure lower and excessive smoking raised it. Edwards, McKeown and Whitfield[18] found in a group of men 60 years and over that mean arterial pressures were rather lower in men smoking cigarettes than in non-smokers, and the means were inversely related to the number of cigarettes smoked daily. Similar conclusions were suggested by observations of Karvonen and his colleagues[19]. In the present data, systolic blood pressure of heavy smokers was higher in farm village A. It is possible that excessive consumption of tobacco may be an indication of heightened emotional tension as Russak and Zohman[20] have suggested.

B. General consideration

Kondo[21] has investigated a number of villages in Japan with abnormally high or low longevity rates (proportion of total population which is aged 70 and over). He found that the rate was low in villages where vegetables were scarce and villagers ate a great deal of rice. The longevity rate is inversely correlated with death rate from cerebral haemorrhage in middle life (30–59)[22].

This at first sight appears hard to reconcile with the prescription in some western countries of Kempner's rice-fruit diet[23] for the treatment of hypertension. However, this diet contains about 2,000 calories, 20gm of protein and less than 5 gm of fat from rice, less than 1 gm of NaCl, a fair amount of sugar and fruit juice and 5,000 I.U. of vitamin A, 1,000 I.U. of D. 5 mg each of B_1 and B_2, 25 mg of nicotinic amide, 2 mg of Ca pantothenic acid etc. These vitamins,

TABLE I. Nutritional Intake per Person per

Bloc of rural districts	Calories	Protein %	Fat %	Carbohydrate %	Calcium mg
Tohoku, Hokuriku, Koshinetsu	2,700	83.4	19.5	466	386
Hokkaido	2,630	89.7	22.7	451	449
Kanto, Tokai	2,410	74.2	20.8	406	349
Kinki, Chugoku	2,404	73.1	16.7	421	382
Shikoku, Kyushu	2,404	76.3	16.6	402	408
Total of rural districts	2,461	76.2	18.9	419	377
Total of urban districts	2,390	77.8	23.6	381	343

especially the B complex, are very deficient in the diet of rural districts in Japan, especially in the north-east area.

Koyanagi and Hareyama[24] demonstrated in rats that the hypertensive state which is normally caused by an excessively salty diet, can be delayed by the addition of riboflavin or pantothenic acid.

Soda, Yuasa and Oiso[25] have concluded from data in the National Nutritional Survey in Japan that the larger the intake of vitamins B_1 and B_2 the higher are both systolic and diastolic pressure. This conclusion was reached after comparison of high and low vitamin intake groups in the farming population of 20 districts. Even in the high intake group the vitamins were not of course taken therapeutically and the amount of vitamins in the natural diets in these areas never aproach pharmaceutically large doses.

As a most important source of the vitamin B group is cereals, it would be expected that the greater the quantity of cereals consumed, the larger would be the vitamin intake. It has to be remembered, however, that the polished rice which most Japanese eat, especially in rural districts, is deficient in vitamins of the B group. It is well-known that those vitamins are components of the enzymes or co-enzymes which are necessary for oxidation and decomposition of carbohydrates in vivo. Carbohydrates are a most important source of calories especially in the rural districts in Japan. Therefore, the greater the amount of rice consumed, the greater is the deficiency of B vitamins.

Vitamin B group requirements should be considered in relation to total calory intake from carbohydrate. In Table I vitamin B intake per 1,000 Cal is compared for 5 rural districts in Japan (data from the National Nutritional Survey in 1954), Tohoku (north-east)-Hokuriku-Kôshinetsu district with the higher polished rice intake shows the lowest vitamin B_1 intake per 1,000 Cal.

Ishiguro, Kaneta and Shiraishi[26] measured blood nicotinic acid in inhabitants of fishing village D where death rate from cerebral haemorrhage is low and the inhabitants consume less rice, and of farm village A where the death rate is

Day in 5 Rural Bloc (1954, Japan)

Phosphorus mg	Iron mg	Vitamin A I.U.	Thiamine mg	Riboflavin mg	Vitamin C	Thiamine per 1000 Cal
1,792	15.8	3,107	1.03	0.75	83	0.38
1,931	14.6	3,324	1.04	0.76	78	0.40
1,810	16.4	2,880	1.30	0.68	74	0.54
1,873	16.9	2,305	1.05	0.59	74	0.44
1,945	15.6	2,110	1.08	0.62	77	0.45
1,851	16.2	2,640	1.15	0.66	76	0.47
1,775	15.8	3,047	1,08	0.65	74	0.45

higher and more rice is consumed. They found a higher level of nicotinic acid in the blood of the inhabitants of fishing village D than of farm village A, while for total serum cholesterol the reverse was true.

In the usual nutritional survey the amount and types of foodstuffs consumed by families in a randomly selected district are estimated and vitamin intakes are then calculated from standard tables of food composition. Kaneta and Meguro[27] directly measured the amount of vitamin B_1, B_2 and water in meals of university hospitals and compared the value with that calculated from the tables. They found that the calculated value of vitamin B_1 varied from 30% to 300% of the direct measure. The difference was less for vitamin B_2 than vitamin B_1, although the water content was almost the same.

It is certain that farm villagers, especially in the north-east of Japan, take a great deal of salt with their simple rice diet [Sasaki[28]] and it seems that there is some relation between salt intake and hypertension, as Dale and Love[29] have reported. However, the underground workers of coalmine G who take about 10 gm more salt than the surface workers (to prevent heat cramp) show a lower blood pressure level. This may be due to the excessive perspiration associated with heavy work under the conditions of high temperature and humidity.

V. Effect of Social and Economic Circumstances

According to Japanese National Statistics there are differences of corrected death rate from cerebral haemorrhage in different occupation groups. These differences can probably be interpreted in terms of differences in the blood pressure level in relation to occupation, age and sex.

When blood pressure levels of primary and middle school teachers and farm villagers in the same Miyagi Prefecture are compared significant differences are found[13]. In this case there appears to be no difference in body build (Pirque's index) among the professional groups. Geographic differences of blood pressure level probably would also be found in school teachers, and even larger differences in farm village dwellers.

Most farm village dwellers are of course farmers. But the non-farmers in those villages have blood pressure levels very similar to the farmers. For example, non-farmers of farm village A — lignite-miners, retailers, public officials etc. — showed no difference in blood pressure levels compared to farmers[2].

Two explanations have been suggested for the higher blood pressure level found among farm villagers, particularly in the north-east. The first is that it is related to the physical demands of their work; the second, and more likely explanation, is that it is due to their living conditions and in particular to an inadequate diet.

Itô[30] demonstrated from 1950 data for 46 prefectures a correlation between the middle-aged (30–59) death rate from cerebral haemorrhage and both total

area of the paddy fields (male $r=+0.609$, female $r=+0.552$; respectively $P<0.001$), and estimated rice yield (male $r=+0.492$, female $r=+0.511$; respectively $P<0.001$). The correlation was again demonstrated by Suzuki[3] with

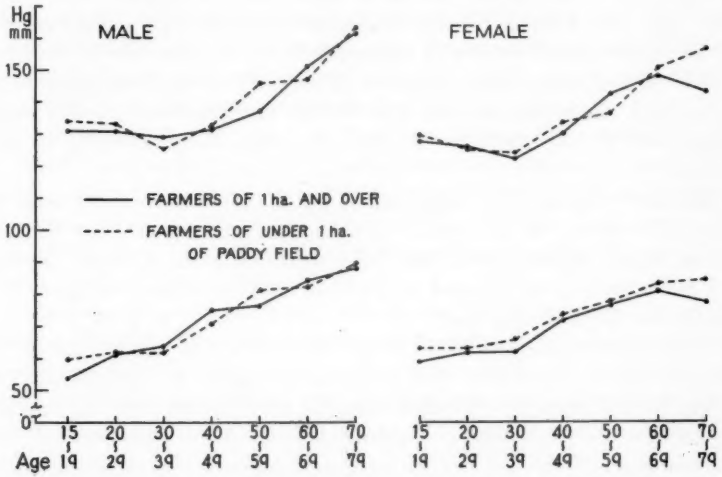

Fig. 10. Comparison of blood pressure levels between farmers grouped by the area of their paddy fields in farm-village A.

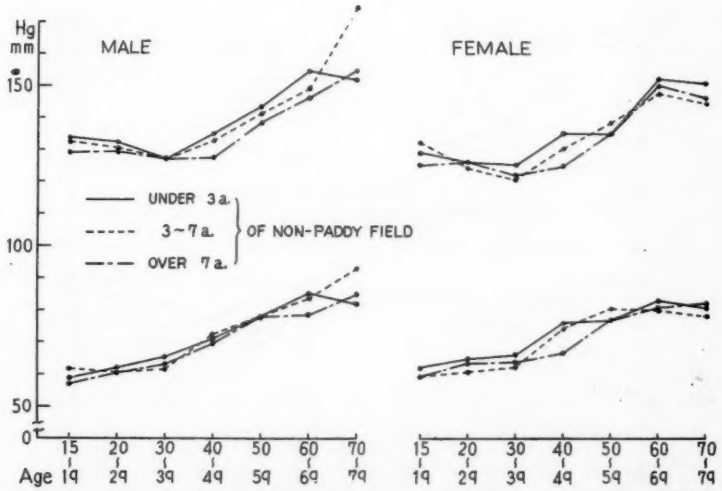

Fig. 11. Comparison of blood pressure levels among farmers grouped by the area of their non-paddy fields in farm-village A.

data for 1955. These results relate to observations which take the prefecture as a unit. When the unit chosen is the family, the results are somewhat different.

Ohba[29] in her investigation on farm village A found no great difference of blood pressure levels between families farming 1 ha. and over and those farming under 1 ha. – the diastolic pressure of females in families with under 1 ha. was a little higher ($P<0.05$), as shown in Figure 10. However, when only dry field area – which is very small in extent compared to the paddy fields – was considered, the families with the greater farming area showed in males lower systolic and in females lower systolic and diastolic pressures (respectively, $P<0.05$), as shown in Figure 11.

Meguro[31] demonstrated a significant difference of blood pressure level in farm village B in relation to area under vegetable cultivation ($P<0.02$). The group with the bigger vegetable field area had lower blood pressure levels. A similar trend had already been reported by Ito[30] in relation to farm-villagers in the Aomori and Akita Prefectures.

These observations suggest that the effect of diet is perhaps greater than that of the physical demands of work, which are lighter in vegetable (dry) field farming than in paddy (wet) field farming. It is of interest that the correlation between area under vegetable cultivation and the blood pressure level of the cultivator is significant, but not the correlation between area under rice cultivation and blood pressure level. There appears to be no relation between blood pressure level and economic status as measured by total area under cultivation.

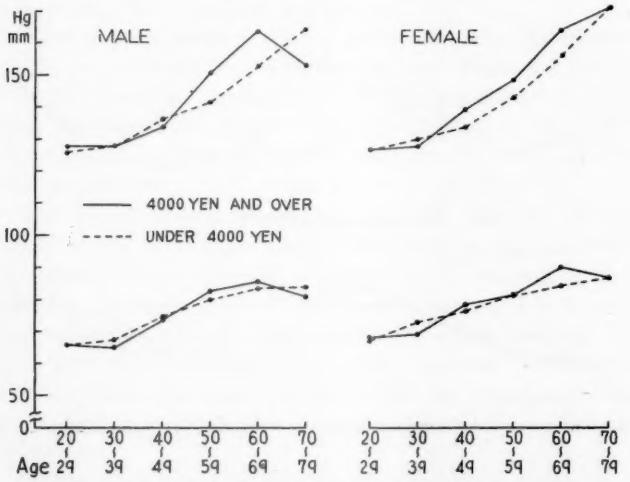

Fig. 12. Comparison of blood pressure levels between 2 groups of provincial supertax amount in farm-village B.

Perhaps even more significant is the fact that a tendency to high blood pressure is found in school children who are not yet working. School children from farm-villages in the north-east of Japan show a higher blood pressure levels than children from other districts[32,33,34,35].

We compared the blood pressure level of about 450 farmers aged 15–79 years in village A before and after the busy season, and we could not find any rise in blood pressure level[36]; in fact there was a tendency for it to fall, although this was probably due to the fact that the temperature was higher.

In farm village B blood pressure levels were compared in relation to provincial tax in two groups; one paying 4,000 yen and over a year and another paying under 4,000. Although in males aged 50–69 and in females aged 40–69 systolic blood pressure level was higher for the former than the latter (respectively, $P<0.01$, $P<0.02$), there was no significant difference in probability product for all age groups taken together (Fig. 12).

In the same farm village B blood pressure was compared between two groups divided according to possession of a radio or subscription to a newspaper. There were not many families without a radio, but there were many who did not take a newspaper. Males aged 50 and over who were newspaper subscribers showed a slightly higher systolic pressure than non-subscribers ($P \fallingdotseq 0.05$), as shown in Fig. 13.

We examined medically 85 members aged over 19 of families receiving social care in a farm village near village A. We could find no difference of blood pres-

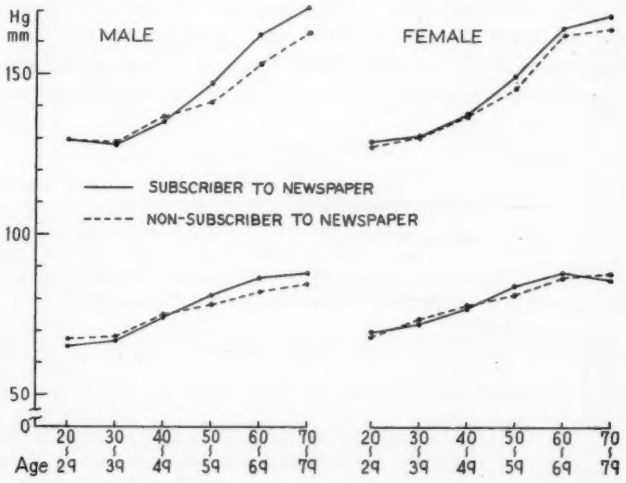

Fig. 13. Comparison of blood pressure levels between subscriber and non-subscriber to newspaper in farm-village B.

sure level between the members of social care families and the general population of the village A.

Generally speaking the living conditions of inhabitants in these farm villages are relatively uniform and the effect of socioeconomic conditions on blood pressure

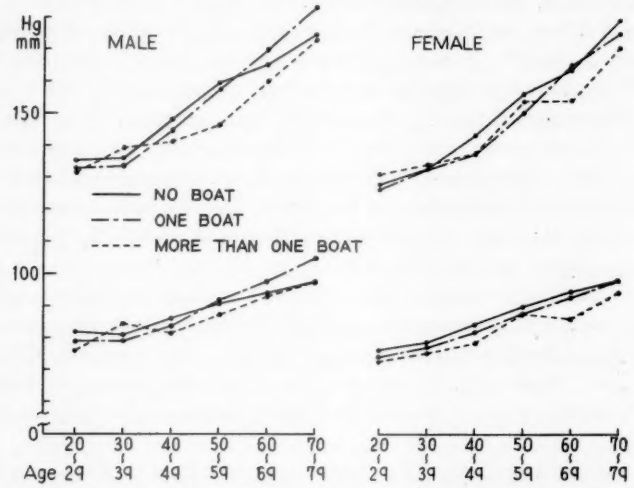

Fig. 14. Comparison of blood pressure levels among fishers grouped by possession of boats in fishing-village D.

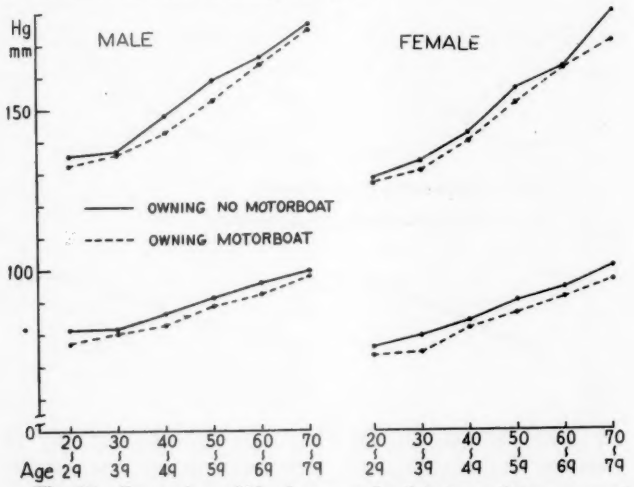

Fig. 15. Comparison of blood pressure levels between fishers grouped by possession of motorboat in fishing-village D.

levels seems to be slight.

Findings in relation to families in fishing village D are of some interest. In shallow sea cultivating, boats of under 3 tons are used. Blood pressure levels of the group without a boat were higher for males (systolic, $P<0.01$) and females (diastolic, $P<0.01$) than of the group with two or more boats (Fig. 14). Recently many fishermen have fitted motors to their boats. As shown in Figure 15 fishermen with motor boats have lower blood pressure levels than those without (in male systolic $P<0.01$ and diastolic $P<0.05$; in female both systolic and diastolic $P<0.05$).

The reasons for these differences are not clear. They are perhaps more likely to be due to economic circumstances, which decide how many boats a family owns, than to the protection against exposure to cold afforded by motor boats, for they were present among the females as well as the males.

SUMMARY

Hypertensive tendencies in the rural inhabitants of north-east Japan are discussed in relation to data collected on the Pacific side of that area. Effects of environmental conditions on blood pressure level are considered under 3 headings; physical environment, diet and socioeconomic circumstances. It is reasonably certain that pressure is inversely related to temperature, but the effect of noise at place of work appears to be unimportant although it can cause a physiological rise. With regard to diet, the consumption of a large amount of polished rice with few vegetables but much salt seems to be a factor increasing the prevalence of hypertension and cerebral haemorrhage in these districts. The effects of socioeconomic circumstancesis less clear, perhaps because the extent of socioeconomic variation is limited in these rural districts. Further investigation of the factors influencing the hypertensive tendency in these areas is needed.

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Cardiac Disturbances due to CO Poisoning in Experimental Animals

I. Electrocardiographic Changes due to CO Poisoning and Those under the Influences of Fluid Infusion

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Experimental and clinical studies on CO poisoning have been repeatedly reported by many pathologists as well as by electrocardiologists. In 1865, Klebs[1] first described abnormal histological findings in CO poisoning. His observations were confirmed experimentally by Christ[2] and Killick[3], and then by Ehrlich *et al.*[4] recently. They observed similarly abnormal findings, namely hemorrhages, degenerative alteration and necrosis in acute CO poisoning, and degenerative, progressive and inflammatory changes in subacute and chronic poisoning. Electrocardiographic changes due to CO poisoning have been reported by many authors. In 1921, Haggard[5] precisely reported abnormal findings of E.C.G. in experimental animals. His observation gave an impetus to experimental and clinical studies on CO poisoning. The most commonly observed changes of E.C.G. were divided into three kinds by previous investigators. The first was displacement of S-T segment. The second was of T wave, namely inversion of T, nagative T, elevation of T or flat T. The third was A-V block.

Wieland[6] described recently that abnormalities in E.C.G. showed the good correlation with pathological changes depended upon the severity of CO poisoning in experimental animals. However, Stearn *et al.*[7] stated that even in the lethal dose of CO poisoning, remarkable changes were missing in E.C.G.

The author observed changes of E.C.G. during and after the CO gas inhalation and maintains that in CO poisoned animals, the latent disturbances of the heart, which were otherwise not detectable, became recognizable in the tracing of E.C.G. after the animals were infused some amount of fluid, which gave an unbearable burden to the circulatory system.

MATERIAL and METHOD

All experiments were performed upon rabbits with about 2 kg weight.

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Experimental courses were classified into three stages. The first is the inhalation of CO gas and the tracing of E.C.G. The second is the fluid infusion 24, 48 or 96 hours after the cessation of gas inhalation and the tracing of E.C.G. The third is the microscopic examination of the hearts.

Inhalation of CO mixed gas : The tracheae were incised under urethan anesthesia and connected to bags by tracheal cannulas in which the non-rebreathing bulbs were set. The animals were set to inhale naturally the gas filled in the bags. The gas in the bags only passed out through non-rebreathing bulbs and did not return back to the bags. The gas was the mixture of room air and 0.3 or 1% of fresh CO gas. The animals inhaled for a certain period the mixture gas from the bags and the bag was taken off to inhale the room air afterwards.

Electrocardiogram : All electrocardiograms were recorded in the lead II.

CO-Hb level : CO-Hb concentrations in blood were determined by the Wolff-Schwerd[8] method.

Infusion of fluid : The fluid was Ringer-Locke solution added 12% bovine hemoglobin. The infusion was performed 24, 48 or 96 hours after the cessation of CO gas inhalation. The fluid kept at 37°C, was infused to the animals at the rate of 20 ml. per kg and 10 ml. per minute. Before and after the infusion, E.C.G. was traced.

Blood pressure and respiration : The blood pressure was recorded on a kymograph from the femoral artery by a mercury manometer and time-courses of the respiration were recorded on the same kymograph from the trachea.

Microscopic examination : The hearts were examined microscopically by formalin fixation, paraffin imbedding, hematoxyline-eosin staining and Van Gieson's staining.

RESULTS

I. Influences of CO gas inhalation

CO-Hb level (Table I) : The CO-Hb concentration in blood of the poisoned animals reached from 33.9 to 42.5% as the inhalation of 0.3% CO gas lasted for 30 minutes and from 36.6 to 43.9% as the inhalation of the same gas lasted for 60 minutes. The elevation of CO-Hb level during the gas inhalation was more prompt for the first 30 minutes than the following 30 minutes. After the gas inhalation ended, the CO-Hb levels descended promptly. The levels descended by 22.1 to 25.5% for the first 30 minutes in the animals poisoned with the gas inhalation which lasted for 30 minutes, and by 14.8 to 19.8% in those poisoned with the gas inhalation lasting for 60 minutes. The drop of CO-Hb level for the first 30 minutes after the cessation of gas inhalation was more prompt in animals poisoned with the gas inhalation which lasted for 30 minutes than in those for 60 minutes. These good examples were Nos. 30, 33 28, and 29. These four animals showed nearly the same value in the end of gas inhalation and the

TABLE I. CO-Hb Level during and after G.I.

Animal No.	CO % in the gas	Duration of G.I.	CO-Hb level							
			30′ after S.G.I.	60′ after S.G.I.	30′ after C.G.I.	Reduction for first 30′	60′ after C.G.I.	Reduction for next 30′	120′ after C.G.I.	Reduction for latter 60′
26	0.3	30	37.8		12.3	−25.5	3.6	− 8.7		
28	0.3	30	36.8		13.5	−23.3	7.8	− 5.7	3.6	−4.2
29	0.3	30	39.2		17.1	−22.1	5.8	−11.3	4.4	−1.4
31	0.3	30	40.8		16.0	−24.8	5.3	−10.7	3.1	−2.2
27	0.3	60	42.6	43.9	25.0	−18.9	14.9	−10.1	7.3	−7.6
30	0.3	60	33.9	36.6	19.2	−17.4	8.9	−10.3	3.5	−5.4
33	0.3	60	36.3	38.4	23.6	−14.8	11.1	−12.5	5.1	−6.5
37	0.3	60	40.0	41.6	21.8	−19.8	8.9	−12.9	4.1	−4.8

S.G.I. : Start of the gas inhalation.
C.G.I. : Cessation of the gas inhalation.

former two animals showed more prompt decrease of CO-Hb levels than the latter two animals. For the next 30 minutes namely, from 30 to 60 minutes after the cessation of the gas inhalation, all animals showed almost similar decreases, that was 10.1 to 12.9%. 120 minutes after the cessation of gas inhalation, all animals showed values of 3.1 to 7.3%.

Influences on E.C.G. (Table II): Changes of E.C.G. during the gas inhalation were seen in the heart cycle and the S-T segment or the T wave. In the group poisoned with the inhalation of CO gas for ten minutes, the cycle was cut by 5 to 55 msec. in interval in five animals out of seven and prolonged by 10 to 70 msec. in the other two. 30 minutes after the cessation, it was recovered except one animal (No. 17), which was remained at the prolonged cycle.

In the group of 0.3% CO gas inhalation for 30 or 60 minutes, the cycle changed variously for the first ten minutes. Many animals except three showed gradual prolongations of the cycles. In two animals (Nos. 32 and 36), the cycle did not change initially and was prolonged afterwards gradually. In one animal (No. 29), it was prolonged promptly in eariler stage and reduced afterwards. After the cessation of gas inhalation, the cycles prolonged during the gas inhalation were reduced except for two animals (Nos. 29 and 36). These excepted animals showed the prolongation of 25 to 60 msec.

The height of R wave changed variously in the group of 0.3% CO gas inhalation for 30 or 60 minutes and was constant in the group of 1% CO gas inhalation. There were no definite changes.

Changes in the S-T segment and R wave: In the group of 1% CO gas inhalation for 10 minutes, five animals out of seven showed no changes. Of two animals, No. 13, showed the elevation of S-T and coronary T and the other (No.

TABLE II. Heart Rate and Potential of R Wave during and after G.I.

Animal No.	CO %	Duration of G.I.*	Heart cycle (msec.) and potential of R (mV.)								Changes of E.C.G. during G.I.	
			Before G.I.	After S.G.I. (min.)						After C.G.I.		
				10	20	30	40	50	60	30	60	
13	1	10	230	200						230		Elevation of S-T, coronary T	Died after 17.5 hours
			0.6	0.4						0.35			
15	1	10	280	225						230		None	Infused
			0.7	0.7						0.8			
16	1	10	230	250						250		Flat T	Died after 19.5 hours
			0.5	0.4						0.5			
17	1	10	230	240						250		None	Died after 8 hours
			0.9	0.9						0.9			
19	1	10	230	220						220		None	Infused
			0.45	0.4						0.4			
22	1	10	230	300						230		None	Died after 18.5 hours
			0.7	0.7						0.9			
23	1	10	210	195						215		None	Died after 14 hours
			0.7	0.7						0.7			
25	0.3	30	215	190	210	220				205	200	None	Infused
			0.4	0.4	0.3	0.3				0.35	0.4		
26	0.3	30	210	210	220	230				230	230	Depression of S-T	Infused
			0.35	0.3	0.2	0.2				0.3	0.3		
28	0.3	30	190	215	270	270				240	240	Elevation of T, tent T	Infused
			0.3	0.3	0.3	0.3				0.35	0.35		
29	0.3	30	200	280	260	245				270	315	Elevation of T	Infused
			0.45	0.7	1.1	0.65				1.1	1.0		
31	0.3	30	210	215	260	280				250	240	None	Infused
			0.65	0.7	0.7	0.7				0.7	0.75		
32	0.3	30	210	210	205	190				190	190	None	Infused
			0.5	0.6	0.5	0.35				0.4	0.45		
36	0.3	30	220	220	200	200				260	250	Depression of T	Infused
			0.4	0.4	0.45	0.4				0.4	0.45		
27	0.3	60	230	240	240	240	250	260	260	215	215	Depression of S-T, flat T	Infused
			0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5		
30	0.3	60	215	210	230	280	280	290	295	250	235	Elevation of T, Coronary T	Infused
			0.45	0.4	0.45	0.45	0.5	0.5	0.6	0.75	0.65		
33	0.3	60	200	200	250	250	260	260	270	220	210	Elevation of T, Coronary T	Infused
			0.55	0.5	0.7	0.7	0.7	0.7	0.7	0.6	0.5		
35	0.3	60	240	250	250	250	260	250	260	260	255	Flat T	Infused
			0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.45	0.45		
37	0.3	60	225	215	225	225	240	250	250	220	240	Depression of T, flat T	Infused
			0.75	0.8	0.7	0.6	0.6	0.65	0.65	0.8	0.8		
38	0.3	60	200	230	240	270	280	280	280	240	240	Depression of T, flat T	Infused
			0.5	0.5	0.7	0.8	0.8	0.7	0.7	0.8	0.7		

*: minutes

No. 13.

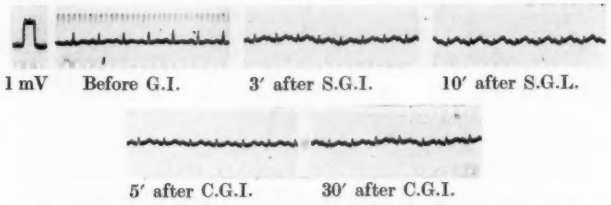

Fig. 1. Change of E.C.G. by the 1% CO gas inhalation; elevation of S-T and coronary T.

No. 16.

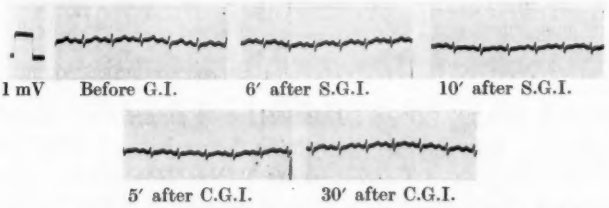

Fig. 2. Change of E.C.G. by the 1% CO gas inhalation for 10 min.; flat T.

No. 27.

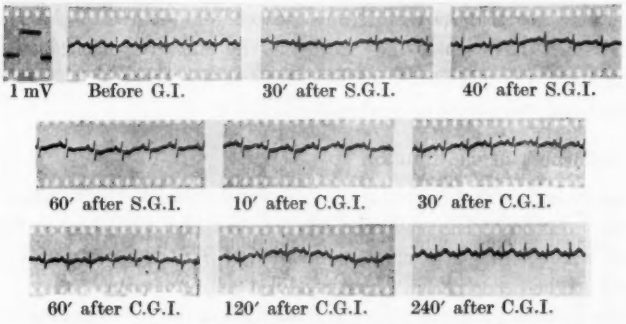

Fig. 3. Change of E.C.G. by the 0.3% CO gas inhalation for 60 min.; depression of S-T and flat T.

16) showed flat T.

In the group of 0.3% CO gas inhalation for 30 minutes, three animals showed no change. Of the remaining four, two animals (Nos. 28 and 29) showed the elevation of S-T and tent T and the other two showed the depression of T or S-T (Nos. 26 and 32).

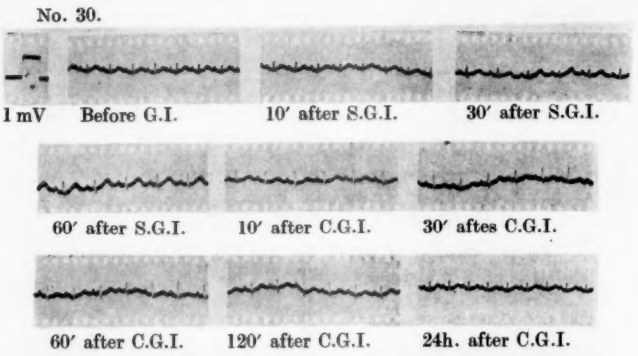

Fig. 4. Change of E.C.G. by the 0.3% CO gas inhalation for 60 min.; elevation of T and coronary T.

In the group of 0.3% CO gas inhalation for 60 minutes, all animals showed changes of E.C.G. Four animals showed the depression of T or S-T and flat T, two showed the elevation of T and coronary T (Nos. 30 and 33).

As described above, there were peculiar changes of E.C.G. in the majority of animals poisoned with the CO gas inhalation lasting for 30 or 60 minutes.

Changes of E.C.G. which were seen during CO gas inhalation disappeared soon after the cessation and the E.C.G. was entirely normal after 24 hours.

There were no intimate correlations between the degree of changes seen in E.C.G. and the CO-Hb level. For example, the animals poisoned with the inhalation of 0.3% CO gas for 30 minutes showed the value of 36.0 to 40.8% in CO-Hb level and there were little changes in E.C.G.

II. Influences of the infusion

Influences on E.C.G. (Table III): In control animals, the common changes seen by the infusion of Ringer-Locke solution to which 12% bovine hemoglobin was added, were sinking of S, slight depression of S-T, depression of T, high voltage R and slight bradycardia. Extrasystoles were not seen.

Acutely poisoned animals with the inhalation of 0.3% CO gas for 60 minutes were divided into two groups. Animals in the one group (Nos. 30, 37 and 38) were infused the solution 96 hours after the cessation of gas inhalation and those in the other group were infused 48 hours later. In the former group, all animals showed extrasystoles, which were respectively regular extrasystoles in No. 30, pararhythmia and multifocal extrasystoles in No. 37 and bigeminy in No. 38. In the latter group, two animals out of three showed multifocal extrasystoles (Nos. 33 and 35) and the other showed only bradycardia (No. 27).

Animals poisoned with the inhalation of 0.3% CO gas for 30 minutes were

TABLE III. Changes after the Infusion

Animal No.	CO % in the gas	Duration of G.I.*	Interval G.I.-Inf.**	Changes of E.C.G.	Blood pressure, mmHg					Respiration, min.				
					Before Inf.	After the Inf.				Before Inf.	After the Inf.			
						just	10′	30′	60′		just	10′	30′	60′
15	1	10	24	Monofocal extrasystole	130	145	144	140	120	28	34	36	40	50
19	1	10	24	Monofocal extrasystole → fibrillation	116	88				32	38			
25	0.3	30	48	Multifocal extrasystole, bigeminy	80	104	110	115	108	24	16	24	33	22
26	0.3	30	48	Multifocal extrasystole, bigeminy	95	116	110	102	90	30	30	36	40	30
36	0.3	30	48	Bradycardia, A-V block, inversion of T	94	49				27	4			
28	0.3	30	96	Bradycardia	80	138	145			33	28	29		
29	0.3	30	96	Sinking of S	93	70	140	128	155	27	20	25	25	31
31	0.3	30	96	Remarkable elevation of S-T, negative T	83	112	127	116	103	25	33	27	26	28
32	0.3	30	96	Remarkable depression of S-T	100	146	169	110	45	48	53	58	40	25
27	0.3	60	48	Bradycardia	100	112	138	120	110	28	30	33	29	30
33	0.3	60	48	Multifocal extrasystole	90	107	130	160	34	34	30	35	29	30
35	0.3	60	48	Pararhythmia, multifocal extrasystole	168	105	132	116	110	25	28	29	31	40
30	0.3	60	96	Monofocal extrasystole	99	112	154	145	134	37	32	36	32	35
37	0.3	60	96	Multifocal extrasystole, pararhythmia, tent T	92	55	94			29	28			
38	0.3	60	96	Monofocal extrasystole, escaped beat, bigeminy	95	55	130	143	132	24	5	50	45	40
14	Control			Slight bradycardia	100	120	130	112	182	18	20	19	27	34
18				Sinking of S	95	124	138	122	124	23	17	18	24	27
20				Slight depression of S-T	88	100	104	116	114	38	54	48	52	40
21					90	98	115	110	115	21	20	27	27	23
40				Elevation of R	70	97	98	100	76	26	25	33	23	24
41				Depression of T	75	88	110	110	86	28	24	30	27	23

*: Minutes, **: Hours
G.I.: Gas inhalation
Inf.: Infusion of fluid

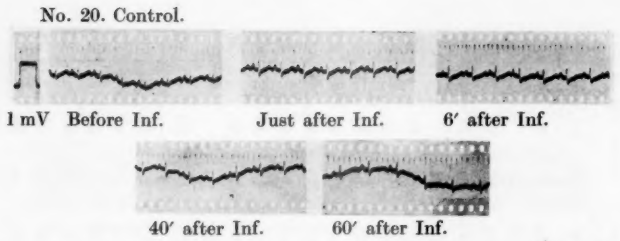

Fig. 5. Change of E.C.G. after the infusion in control animal; without CO gas inhalation.

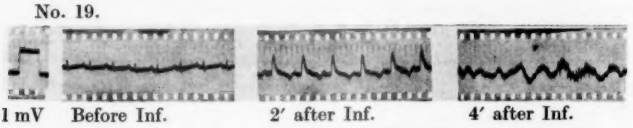

Fig. 6. Fibrillation was produced in this animal poisoned with 1% CO gas inhalation for 10 min. and infused 24 hours later.

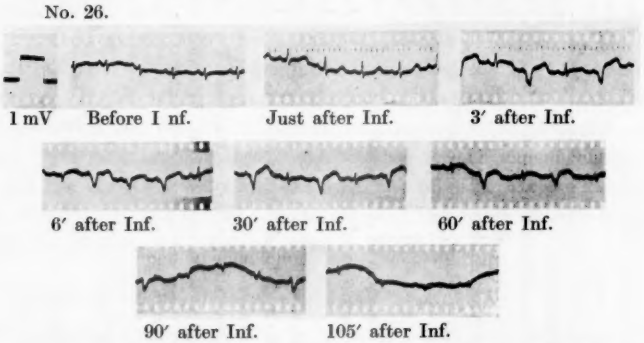

Fig. 7. Multifocal extrasystole and bigeminy were shown in No. 26 animal poisoned with 0.3 % CO gas inhalation for 30 min. and infused 48 hours later.

investigated similarly as described above. In the one group, animals were infused the solution 48 hours after the cessation of gas inhalation and in the other group, those infused 48 hours later. In the former group, all animals (Nos. 28, 29, 31 and 32) did not show extrasystoles but No. 28 showed remarkable bradycardia and immediately fell into shock, and Nos. 31 and 32 showed remarkable displacement of S-T and negative T. In the latter group, two animals (Nos. 25 and 26) showed multifocal extrasystoles and other one (No. 36) showed A-V block and inversion of T.

No. 33.

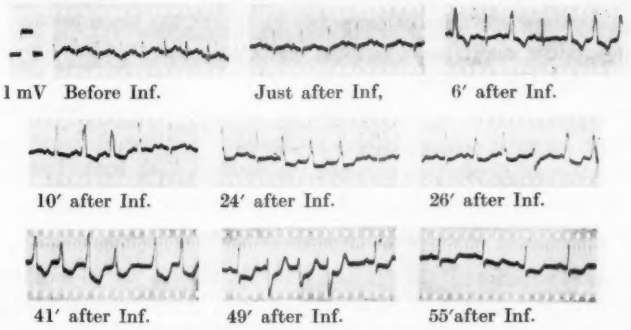

Fig. 8. Multifocal extrasystole was shown in No. 33 animal poisoned with 0.3 % CO gas inhalation for 60 min. and infused 48 hours later.

No. 37.

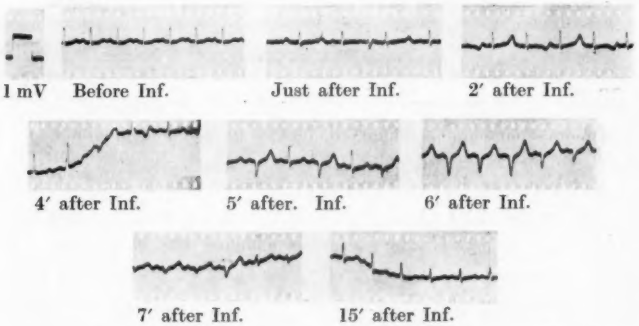

Fig. 9. Multifocal extrasystole, pararhythmia and tent T were shown in No. 37 animal poisoned with 0.3% CO gas inhalation for 60 min. and infused 96 hours later.

Many of the animals poisoned with 1% CO passed away within 24 hours and survived animals (Nos. 15 and 19) were infused the solution 24 hours later. Both animals showed multifocal extrasystoles and in No. 19 fibrillation was produced after 4 minutes.

Influences on blood pressure and respiration: Just after the infusion, the blood pressure was elevated by 8 to 29 mm Hg in control animals and this continued for 10 to 30 minutes. In acutely poisoned animals, the initial elevation of blood pressure was found in ten out of fifteen and the highest value of it reached 58 mm Hg. The remaining five animals did not show the elevation. No. 19 produced the fibrillation and No. 36 fell into shock. In two animals

(Nos. 29 and 38), the blood pressure dropped just after the infusion and was followed with a prompt elevation afterwards. In No. 37, the blood pressure dropped in the latter half of the infusion and was temporarily elevated but dropped again 18 minutes after the infusion.

No correlations were found between the changes of blood pressure and the E.C.G. except in some animals, which produced fibrillation or fell into shock.

The number of respiration changed variously in both control and poisoned animals. The definite patterns could not be obtained.

III. Microscopic examination of the hearts

Animals poisoned with the inhalation of 1% CO gas for 10 minutes showed small hemorrhages, slight infiltration of leucocytes around vessels and hyalinous appearances of individual muscle fibers. Remarkable degenerative alteration and necrosis were not seen.

Animals poisoned with the inhalation of 0.3% CO showed necrosis, infiltration of leucocytes, histiocytes and fibrocytes, which were more remarkable in animals surviving for 96 hours than in those surviving for 48 hours.

DISCUSSION

The CO-Hb levels in the blood reached 33.9 to 42.5% by the inhalation of 0.3% CO gas for 30 minutes and 36.6 to 43.9% by that for 60 minutes. The elevation of CO-Hb level is prompt for the first 30 minutes during the gas inhalation and becomes slow afterwards. This process is similar to the curves of CO-Hb level reported by Henderson *et al.*[9] and Kalthoff[10]. But the observation showed that the CO-Hb levels never reached the saturation value. The CO-Hb levels decreased by 14.8 to 25.5% for the first 30 minutes after the cessation of gas inhalation and the decrease is prompt initially and becomes slow afterwards. This process is similar to that reported by May[11]. From results, it is noted that the decrease of CO-Hb level after the cessation of gas inhalation depends upon the duration of CO gas inhalation; that is, the decrease is more remarkable in animals poisoned with gas inhalation for a shorter period. The author infers that the CO reserve, which is resulted from the combination of CO to myoglobin and/or cytochrome c, exists together with the lowering of respiratory function. This idea may be supported by the results described above and the fact that it requires a certain time to produce the E.C.G. changes.

The E.C.G. during CO gas inhalation was observed in twenty animals. Twelve animals showed changes and eight showed none. The changes were noted in all animals poisoned with the inhalation of 0.3% CO gas for 60 minutes, in four animals out of seven which were poisoned with the inhalation of 0.3% CO for 30 minutes and only two animals out of seven which were poisoned with the inhalation of 0.1% CO for 10 minutes. The E.C.G. changes seen in these animals

were elevation or depression of the S-T segment and coronary, tent or flat T wave. The depression of T was described by Christ[3], Breu[12] and Groetschel[13]. The depression of T was mentioned by Ehrlich[4] and Thiel[14]. Other authors reported the inversion of T and A-V block[15]. Stearn *et al.*[7] also stated that even in the lethal dose of poison, marked changes in E.C.G. might be missing. On the other hand, Wieland[6] described that abnormalities in E.C.G. showed the good correlation with pathological changes in experimental animals. The changes observed by the author resemble those obtained by previous investigators. The fact that no electrocardiographic changes were seen in some of animals poisoned with CO, may support the statement of Stearn *et al.*

Changes of the S-T segment and the T wave observed are explained by anoxia because they resemble those seen in anoxic circumstances and because of the nature of CO. In my experiments, all animals poisoned with CO gas inhalation for 10 or 30 minutes did not show any changes. From these results, the author infers that it may require a definite time duration, for which high concentration of CO-Hb level is maintained, to produce the changes in E.C.G. The disturbances in heart muscles may be induced by the combination of CO to myoglobin and/or cytochrome c. The CO-Hb level may be only an indicator showing an extent of CO gas inhalation.

The electrocardiographic changes seen during the gas inhalation disappear soon after the cessation of gas inhalation and the E.C.G. is entirely normal after 24 hours. Those acutely poisoned animals were infused Ringer-Locke solution added 12 % bovine hemoglobin 24, 48 or 96 hours after the cessation of inhalation. The disorders of E.C.G. appeared by the infusion. In control animals, the changes were slight and extrasystoles were not seen. In acutely poisoned animals, the changes were remarkable, namely, nine animals out of fifteen showed extrasystoles, one showed A-V block, one showed high degree of bradycardias and the other two showed remarkable displacement of the S-T segment. The disorders of E.C.G. are peculiar in the poisoned animals.

It is conceivable that the disturbances induced by CO gas inhalation remained as latent, although the changes of E.C.G. caused by CO gas inhalation returned to normal, and that weakened hearts were unable to bear the burden of infusion.

Only two animals showed bradycardia or sinking of S but no extrasystoles.

Microscopic changes resemble those reported by many authors[4,15-19]. It is natural that animals which survived for a longer period show more remarkable vital reactions. The abnormal findings in the microscopic examination were observed in animals which did not show the changes in E.C.G. by CO gas inhalation. Accordingly, these results are contradictory to Wieland's statement that the severity of poisoning and electrocardiographic changes were in accord with the pathological changes.

Changes of the blood pressure and the respiration were various and the

definite pattern cannot be obtained.

SUMMARY

Electrocardiographic changes due to acute CO poisoning and those under the influences of the fluid infusion were observed in rabbits.

1) The progresses of CO-Hb level during and after the inhalation of CO gas were similar to those reported by previous investigators. The CO-Hb level never reached the saturation value. The drop of CO-Hb level for the first 30 minutes after the cessation of gas inhalation was more prompt in the animals poisoned with the gas inhalation for a shorter duration than in those for a longer duration.

2) The changes of E.C.G. during the gas inhalation were observed in twelve animals out of twenty. The changes were elevation or depression of the S-T segment and coronary, tent or flat T wave. There was no intimate correlation between the degree of E.C.G. changes and the CO-Hb level. The author infers that it may require a definite time duration, for which high concentration of CO-Hb level is maintained, to produce the changes in E.C.G.

3) Microscopic changes resemble those reported by many authors. Those changes were observed in the animals which did not show the changes in E.C.G.

4) Changes in E.C.G. during the gas inhalation disappeared soon after the cessation of gas inhalation and the E.C.G. returned back to normal after 24 hours. The acutely poisoned animals were infused Ringer-Locke solution added 12% bovine hemoglobin 24, 48 or 96 hours later. The disorders of E.C.G., namely, extrasystoles, remarkable displacement of the S-T segment or A-V block, appeared by the infusion. It is conceivable that the disturbances induced by CO poisoning remained as latent, although the changes of E.C.G. caused by gas inhalation returned to normal and that weakened hearts were unable to bear the burden of the infusion. Thus the cardiac damages must have become manifest.

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Cardiac Disturbances due to CO Poisoning in Experimental Animals
II. Changes of the Heart Excitability due to Acute CO Poisoning

By

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Changes of the heart in CO poisoning have been studied physiologically or electrocardiologically by many authors. But no report has been published concerning the excitability of heart as studied by the author in the present experiments. He presents some findings on the heart excitability utilizing electronic devices similar to those described in a fine monograph "Excitability of the heart" by Brooks and his collaborators[1]. Changes of the heart excitability are serious not only during CO gas inhalation, but also even after its cessation in experimental animals.

MATERIAL and METHOD

All experiments were performed upon mongrel dogs following the Brooks' technique. The animals were anesthetized with pentobarbital sodium of 30 to 40 mg per kg by intraperitoneal injection with supplement of this agent to prevent the spontaneous respiratory movement. While using artificial respiration of pure oxygen under positive pressure by the to and fro apparatus through the canister filled with soda-lime, the pericardium was opened lengthway following the incision to the left fifth intercostal space. Electrodes for driving were firmly attached to the epicardium of right auricular appendage with No. 1 silk threads. After the pacemaker was crushed, the heart was driven at a desirable rate (400 msec. interval, mainly) by thyratron discharge, the strength of which was adjusted to the level approximately twice the threshold. Both stimulating electrodes for testing and recording electrodes embeded in plastique were attached crosswise on the left ventricle in the same manner as driving electrodes. The test stimulus employed was a square wave pulse of which duration was 4 msec. and its amplitude could be varied. With some interval from R wave which was evoked by a driving electric pulse, the strength of a test stimulus was gradually in-

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creased until an extrasystole was produced. The stimulus intensity at this moment was read on the oscilloscope screen by a current measurement device. With these procedures, the strength-interval curves in the cases of both normal condition and acute CO poisoning were plotted. Animals were poisoned with CO by the following procedure. CO gas freshly made was mixed with pure oxygen at the rate of 0.05, 0.25, 0.5 or 1% in the bag, which was connected to the canister. Animals inhaled this gas through the canister filled with soda-lime.

CO-hemoglobin (Hb) level was determined by Wolff-Schwerd method from the blood, which was drawn from a femoral artery at intervals of 15 minutes.

Blood pressure was recorded on a kymograph from the femoral artery by a mercury manometer.

RESULT

The experiments were performed upon twelve dogs. The CO concentration in the mixed gas used in the inhalation for 60 minutes was 0.05 to 1%. The 2.5% CO mixed gas was used in the inhalation for 15 minutes.

Table I. Duration of the Gas Inhalation and CO % in the Gas

Animal No.	CO % in the mixed gas	Duration of the gas inhalation
109, 119	0.05	60 minutes
101, 104	0.25	〃 〃
55, 57, 60	0.5	〃 〃
79, 82, 92	1.0	〃 〃
106, 132	2.5	15 〃
86, 96, 99	Control	

The results are shown in Table II.

The change of the strength-interval curve: In control animals, the absolute refractory periods (A.R.P.) and the relative refractory periods (R.R.P.) were both prolonged as time went by. After about 60 minutes, No. 85 (Fig. 1) and No. 99 showed 5 msec. prolongation in A.R.P. and 15 msec. prolongation in R.R.P., and No. 96 showed a few msec. prolongation in A.R.P. The change of resting threshold was not observed. After about 120 minutes, in all control animals, the refractory periods were prolonged still more, but there was no change in resting thresholds. Thus the strength-interval curves in control animals shifted to the right side and the bounds of those shifts were within 5 msec. after 60 minutes,

TABLE II. Changes of Heart Excitability

Animal No.	CO %	Duration of inhal.*	Time of determination**	A.R.P. (m sec.)	R.R.P. (m sec.)	Resting threshold (mA)	Site of dip	Prol. A.R.P. (mA)	Prol. R.R.P. (mA)	Blood pressure (mmHg)
109	0.05	60	before	132	190	0.15	145			50- 60
			20- 45	143	210	0.15	170	11	20	45- 55
			60- 80	152	225	0.15	180	20	35	40- 45
			120-145	155	230	0.15	185	23	40	40
119	0.05	60	before	130	200	0.14	146			70- 75
			15- 29	135	300	0.14	150	5	0	60- 75
			60- 77	150	210	0.14	170	20	10	60- 65
			105-122	150	220	0.14	175	20	20	60
101	0.25	60	before	140	195	0.30	165			70- 75
			20- 58	145	210	0.30	175	5	15	90
			95-125	148	230	0.35	180	8	35	80- 90
104	0.25	60	before	130	205	0·25	160			80- 85
			19- 41	145	205	0.25	170	15	0	70- 80
			55- 74	150	220	0.25	170	20	15	85- 95
			120-148	160	235	0.25	185	30	30	80- 90
55	0.5	60	before	185	260	0.2	200			80-100
			40- 60	200	270	0.2	210	15	10	90-105
			110-140	200	270	0.25	210	15	10	80- 90
57	0.5	60	before	140	220	0·25	155			90- 80
			10- 50	140	210	0.3	160	0	−10	100- 85
			25-110	150	210	0.3	170	10	−10	70- 80
60	0.5	60	before	160	260	0.25	185			75- 60
			30- 50	175	260	0.25		15	0	70- 85
			65- 88	180	280	0.25		20	20	70- 80
			100-120	185	280	0.25		25	20	70
79	1.0	60	before	165	260	0.3	185			90-105
			55- 77	170	260	0.3	190	5	0	85-100
			103-127	175	260	0.4	200	10	0	70
			145-170	175	260	0.4	200	10	0	75
82	1.0	60	before	160	240	0.25	175			55- 60
			15- 41	170	240	0.25	180	10	0	60- 70
			108-135	170	230	0.3	185	10	−10	60- 70
			163-188	170	230	0.3	180	10	−10	60- 45
92	1.0	60	before	145	195	0.2	155			85- 90
			18- 37	150	195	0.2	160	5	0	80- 90
			50- 70	170	215	0.2	175	25	20	70- 80
			135-160	180	230	0.2	180	35	35	80- 85
122	2.5	15	before	110	175	0.12	115			55- 60
			2- 22	105	175	0.12	115	0	0	50- 70
			50- 73	110	180	0.12	120	5	5	60- 70
			120-148	110	180	0.12	125	5	5	55- 75
			180-213	110	180	0.12	120	5	5	60- 80
106	2.5	15	before	125	190	0.11	140			110-120
			2- 11	115	200	0.11	135	0	10	120
			60- 84	120	200	0.11	135	0	10	110-115
			120-138	120	200	0.11	140	0	10	90-100

* minutes
** minutes after the start of gas inhalation

TABLE II. (Continued.)

Animal No.	CO %	Duration of inhal.*	Time of determination**	A.R.P. (m sec.)	R.R.P. (m sec.)	Resting threshold (mA)	Site of dip	Prol. A.R.P. (mA)	Prol. R.R.P. (mA)	Blood pressure (mmHg)
86	Control		0- 30	115	195	0.1	135			85- 95
			60- 87	120	195	0.1	135	5	0	80- 85
			120-165	120	205	0.1	140	5	10	70- 80
			180-215	125	205	0.1	145	10	10	80- 90
96	Control		0- 20	150	230	0.13	175			110- 90
			50- 70	153	225	0.13	175	5	− 5	70- 90
			100-135	160	220	0.13	185	10	−10	70- 90
99	Control		0- 25	130	210	0.14	155			75- 90
			50- 75	135	210	0.14	160	5	0	80- 90
			110-148	135	215	0.14	160	5	5	85-100

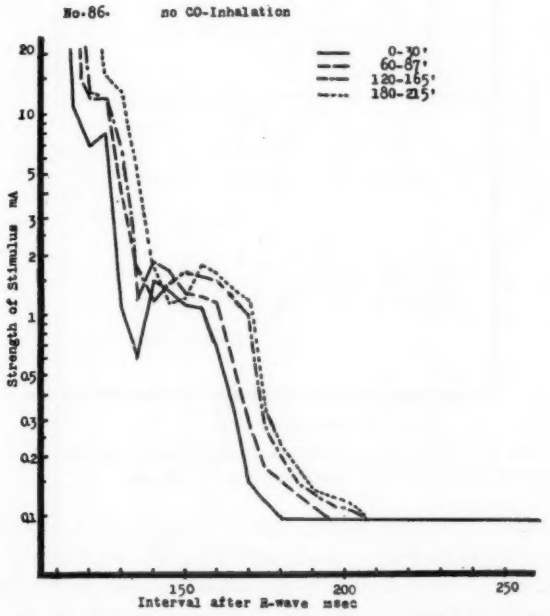

Fig. 1. Change of the heart excitability in control animal as time goes by.

5 to 10 msec. after 120 minutes and 10 msec. after 180 minutes. And the level of resting threshold was kept horizontally.

Due to the inhalation of 0.05% CO gas for 60 minutes, the remarkable prolongation of refractory periods was observed. In two animals (Nos. 109 and

119), the prolongation of A.R.P. was 5 to 10 msec. during the gas inhalation, and 18 msec. after the gas inhalation. The prolongation of R.R.P. was also remarkable in both animals and reached to 35 msec. in No. 109 (Fig. 2). The site of dip shifted to the right and the level of dip decreased remarkably in No. 119. The level of resting threshold was not varied in both animals. Thus the shift of strength-interval curves was remarkable and continued after the cessation of gas inhalation. The degree of shifts was remarkable until 60 minutes after the cessation of gas inhalation, and was at leisure afterwards.

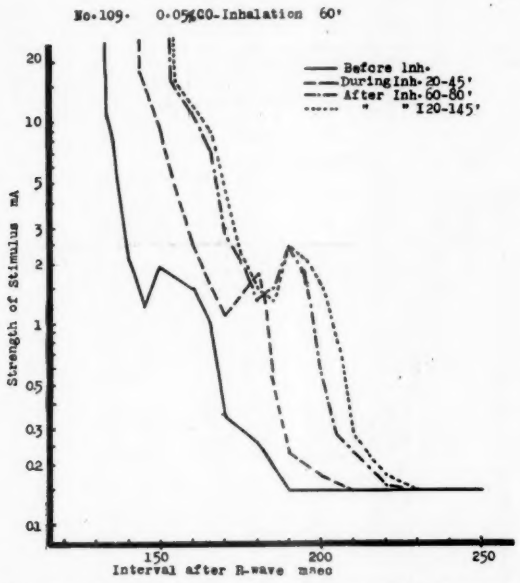

Fig. 2. Change of the heart excitability in No. 109 animal poisoned with 0.05 % CO gas inhalation for 60 min.

The shifts due to the inhalation of 0.25% CO gas for 60 minutes were almost similar to those described above. In No. 104 (Fig. 3), the shift was remarkable and the bound was 15 msec. during the gas inhalation and was 30 msec. after the cessation of gas inhalation. In No. 101, the degree of shift was not remarkable and the bound was within 5 msec. during the gas inhalation and 8 msec. after the cessation of gas inhalation. The level of resting thresholds did not change in No. 104 and was raised by about 0.05 mA in No. 101.

Due to the inhalation of 0.5% CO gas for 60 minutes, the strength-interval curves shifted to the right side similarly. These shifts continued after the cessation of gas inhalation. The prolongation of A.R.P. was 10 to 25 msec. and

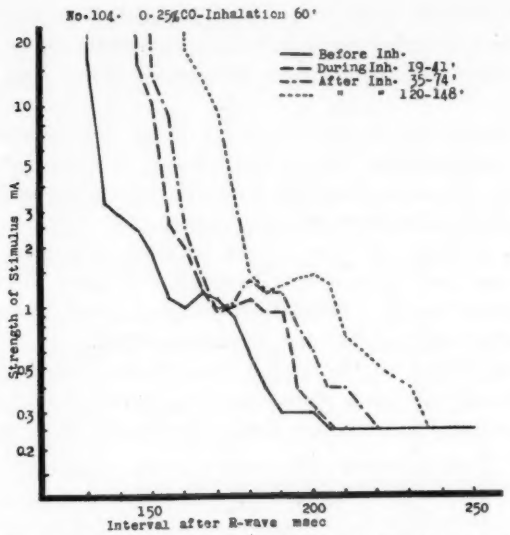

Fig. 3. Change of the heart excitability in No. 104 animal poisoned with 0.25% CO gas inhalation for 60 min.

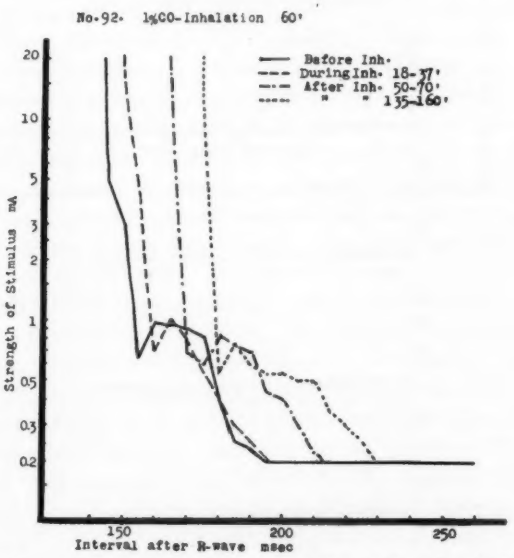

Fig. 4. Change of the heart excitability in No. 92 animal poisoned with 1 % CO gas inhalation for 60 min.

those of R.R.P. were 10 to 20. msec. In two animals (Nos. 55 and 57) out of three, the elevation of resting threshold was observed and in No. 60, the dip which was observed before the gas inhalation disappeared during and after the gas inhalation.

Due to the inhalation of 1% CO gas for 60 minutes, the strength-interval curves showed similar shifts. In No. 92 (Fig. 4), the prolongation of A.R.P. reached 45 msec. The level of resting thresholds did not change in two animals out of three and was elevated in the other animal.

Due to the inhalation of 2.5 % CO gas for 15 minutes, the prolongations of A.R.P. were 5 msec. and those of R.R.P. were 5 to 10 msec. The level of resting threshold did not change. The shift of strength-interval curves in this group was slight and the bound of shift was within the control.

CO-Hb level (Fig. 5): The CO-Hb concentration was determined every 15 minutes. The CO-Hb levels were increasing quickly for 15 to 30 minutes after the start of gas inhalation and slowly afterwards. In 60 or 120 minutes

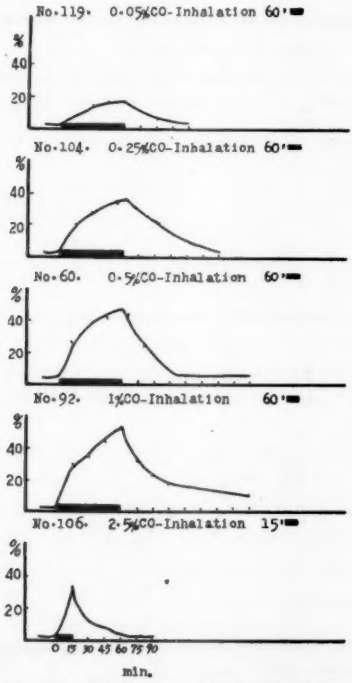

Fig. 5. CO-Hb concentration in blood during and after the CO gas inhalation.

after the cessation of gas inhalation, the levels were nearly the same as before the gas inhalation. The progress of CO-Hb levels was almost similar to one another in animals poisoned with CO gas inhalation in spite of the difference of CO concentration in mixed gas, which was inhaled.

Blood pressure: The blood pressure was observed as a guide of experiments. The blood pressure was lowered to some degree by anesthetic and chirurgic procedures. The elevation and drop of blood pressure during and after the gas inhalation were within 10 to 20 mm Hg except No. 79 in which the blood pressure change reached 30 mm Hg.

DISCUSSION

In control animals, the shift of strength-interval curve to the right is observed and the prolongation of A.R.P. is within 5 msec. In the experiments with the inhalation of 0.05 to 1% CO gas for 60 minutes, all animals show the prolongation of refractory periods and the strength-interval curves are shifted to the right remarkably. The degree of shifts in poisoned animals is nearly or over twice as much as those in control animals. The resting threshold is not varied in control animals and the majority of poisoned animals, but is elevated in a few poisoned animals. Then it is conceivable that the excitability of the heart muscles is decreased in the acutely poisoned animals with the inhalation of CO gas, and this decrease takes place during the gas inhalation and continues after the cessation of gas inhalation, the decrease being remarkable for a certain time after the cessation of CO gas inhalation. Results similar to those shown in my experiments were observed in the cooled heart as well as in the anoxic heart. Haugnauer and Covino[3] observed the shift to the right in the strength-interval curve by cooling experiment, and on the other hand, Brooks *et al.*[3] described the prolongation of refractory period and the elevation of resting threshold at low temperature. The present study shows the result similar to those of the cooling experiment and the findings in the anoxic stage of the heart obtained by Yanagita[4] and Murotsu[5] in our laboratory.

The CO-Hb levels showed increases, which reached nearly 20% in animals poisoned with the inhalation of 0.05% CO gas for 60 minutes and over 40% in those poisoned with the inhalation of 0.5 to 1% CO gas for the same duration. The concentration of O_2-Hb in blood decreases because of the increase of CO-Hb and it is clear that the amount of oxygen supplied to the heart muscles falls off. This principle is the same with hypoxia in anemia or in circumstance of hypo-oxygen. And in cooled hearts there is no lack of oxygen, but heart muscles cannot obtain enough oxygen from blood because of decreased dissociation of oxygen from Hb. It is certain that the abnormal findings under these conditions are attributed to the lack of oxygen in heart muscles. Therefore, the lowering of heart excitability in CO poisoned animals may be originated from anoxia

directly.

For a certain time after the cessation of CO gas inhalation, the animals showed the lowering of heart excitability, which became more remarkable. It may be attributed to disturbances of myoglobin and/or cytochrome c. Enzymatic activities of myoglobin and cytochrome c are due to Fe as well as hemoglobin. This $Fe^{\cdot\cdot}$ combines with CO firmly and transformation from $Fe^{\cdot\cdot}$ to $Fe^{\cdot\cdot\cdot}$ is checked. In CO poisoning, not only hemoglobin but myoglobin and/or cytochrome c combine with CO and after the cessation of CO gas inhalation, CO combined with hemoglobin is removed externally but yet CO combined with myoglobin and/or cytochrome c, remained in heart muscles, because of hard dissociation. Therefore, hypoxia of heart muscles continues after the cessation of CO gas inhalation, and this mechanism is responsible for the lowering of heart excitability, which continues and grows severe even after the cessation of gas inhalation.

Due to the inhalation of 2.5% CO gas for 15 minutes, animals showed little changes, which were within those observed in control animals. It may be due to the CO-Hb level, which is not so high as in the animals poisoned with the inhalation of CO gas for 60 minutes, and the time duration of gas inhalation is a main factor. In the animals poisoned with the inhalation for 60 minutes, the CO-Hb level was more than 40%, but in those for 15 minutes, it was less than 35%. It may require a certain time to make the heart less excitable. The level of CO-Hb may indicate the degree of CO inhalation but does not show the degree of CO poisoning in heart muscles, because there are differences between the affinities of CO to hemoglobin and myoglobin and/or cytochrome c.

It may be concluded that it requires a definite time duration for which the CO-Hb is maintained at a certainly high level, to produce the lowering of the heart excitability.

In the experiment with the inhalation of 2.5% CO gas for 15 minutes, the reduction of A.R.P. was observed. The same results were shown by Murotsu[4] in anemia and Yanagita[5] in low oxygen circumstances. They attributed the reason to the compensatory function in early stage of hypoxemia. The reduction in my experiments may be caused by the same mechanism.

The blood pressure was recorded as a guide for good progress of the experiment. The results described in this paper are limited to the date which did not show remarkable drop. And the elevation over 30 mm Hg was not observed during the CO gas inhalation.

SUMMARY

Changes of the heart excitability were observed in dogs poisoned acutely with the inhalation of CO gas.

1) Due to the inhalation of 0.05 to 1% CO gas for 60 minutes, the remarkable prolongation of refractory periods was observed in all animals and the

elevation of resting threshold was seen in a few animals. The lowering of heart excitability was shown not only during the gas inhalation but after its cessation. Then the strength-interval curve shifted to the right.

2) Due to the inhalation of 2.5% CO gas for 15 minutes, the heart excitability was decreased slightly during the gas inhalation and recovered soon by the inhalation of pure oxygen. Then the absolute refractory period was reduced.

It requires a definite time duration for which the CO-Hb level is maintained at a certainly high level, to produce the lowering of the heart excitability.

3) Lowering of the heart excitability continued and became severe after the cessation of gas inhalation. The disturbances of heart muscles due to CO poisoning may be attributed to disturbances of myoglobin and/or cytochrome c.

4) The reduction of absolute refractory period was observed by the inhalation of 2.5% CO gas for 15 minutes and this may be attributed to the compensatory function in the early stage of hypoxemia.

I wish to thank Prof. Dr. K. Matsuda and Assist. Prof. Dr. T. Hoshi, Department of Physiology, School of Medicine, Tokyo University, for their advices and encouragements.

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Role of Chemical Elements in Eliciting Experimental Cutaneous Calcinosis, Following Pretreatment with Dihydrotachysterol[1]

By

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A cutaneous calcinosis with sclerosis which resembles human scleroderma, has been produced in rats treated during the first few days of life with parathyroid hormone[1)2)] or with dihydrotachysterol (DHT)[3)], a vitamin-D derivative, also known as "AT–10," with a pharmacologic action closely resembling that of parathyroid hormone. The experimental model thus produced not only helps to clarify the pathogenesis of calcifying scleroderma in man — referred to as "sclérodermie calcaire" by French dermatologists — but also enables us to investigate the mechanism of tissue calcification in general, one of the most commonly observed phenomena in physiologic and pathologic states of the biologic field.

Although it has been noted that clinical scleroderma[4)] as well as myositis ossificans[5)] tend to develop at sites of local injury, the occurrence of experimental cutaneous calcinosis is also facilitated in traumatized areas if the animals are optimally pretreated with DHT.[6)] In other words, topical trauma seems to be necessary to promote the production of soft tissue calcification.[7)]

The object of this experiment is to investigate the local effect of various chemical elements acting as chemically traumatizing agents, and to screen their specific characteristics in producing calcification of cutaneous tissue in rats sensitized by treatment with DHT.

MATERIALS AND METHODS

Three hundred forty-four female Sprague-Dawley rats of the Holtzman farm,

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* Fellow of the Rockefeller Foundation.

** Fellow of the Quebec Heart Foundation, Inc.

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with a mean initial body weight of 99 g. (range : 85–108 g.), were subdivided into 86 groups each consisting of 4 rats.

DHT was administered by stomach tube to all groups, at the dose of 50 μg. in 0.5 ml. of corn oil, once daily during the first 4 days. If DHT is given in higher amounts, a slight local trauma, even the injection needle itself, can cause local calcification of the skin. Such effects of nonspecific trauma do not occur when DHT is administered at the level used in the present experiment; but the dose suffices to reveal the specific action of chemical compounds as regards their ability to elicit cutaneous calcinosis. Water soluble inorganic salts and some acetates were employed in the experiment. A few of the compounds used are decomposed by water; in these instances, the solutions were prepared in such a way that the ionic concentrations remained unchanged.

On the 4th day, the substances were injected, in 0.2 ml. of water, subcutaneously, at three different sites of the shaved back, under light ether anesthesia. With the exception of $Fe_2(SO_4)_3$, Ag_2SO_4 and $Al_2(SO_4)_3$, which were given at the concentrations of 0.1 mM/l., 1 mM/l. and 10 mM/l., in order to unify the concentration of cation, all salts were administered at the concentrations of 0.2 mM/l., 2 mM/l. and 20 mM/l.

The animals were killed with chloroform on the 11th day. The skin was dissected and the degree of cutaneous calcinosis was appraised macroscopically in terms of an arbitrary scale of 0 (no lesions), 1 (just detectable lesion), 2 (moderate lesion), and 3 (maximal lesion). In doubtful cases, calcification was confirmed histologically by the von Kóssa stain. The mean degree of calcinosis observed in each group is shown in the table.

RESULTS

Four animals treated with $UO_2(CH_3COO)_2$, one with $Hg(CH_3COO)_2$ and one with Na_2SeO_3 died before the end of the experiment.

The number of substances used in the present investigation was limited; this fact should be kept in mind when evaluating the results.

As shown in Table I, it seems clear that alkali and alkali earth metals do not produce calcification at the concentrations given.

TABLE I. Role of Chemical Elements in Eliciting Cutaneous Calcinosis, Following Pretreatment with DHT

Compound	Cutaneous Calcinosis (scale: 0-3)		
	0.2 mM/l.	2 mM/l.	20 mM/l.
LiCl	0	0	0
NaCl	0	0	0
Na_2SO_4	0	0	0
$NaNO_3$	0	0	0
$NaCH_3COO$	0	0	0

TABLE I (Continued)

Compound	Cutaneous Calcinosis (scale: 0-3)		
	0.2 mM/l.	2 mM/l.	20 mM/l.
KCl	0	0	0
K_2SO_4	0	0	0
RbCl	0	0	0
CsCl	0	0	0
$(NH_4)_2SO_4$	0	0	0
$MgCl_2$	0	0	0
$CaCl_2$	0	0	0
$Ca(CH_3COO)_2$	0	0	0
$SrCl_2$	0	0	0
$BaCl_2$	0	0	0
$LaCl_3$	0.7	2.0	3.0
$CeCl_3$	0.7	2.0	3.0
$UO_2(CH_3COO)_2$	0	0	0
$TiCl_3$	0	2.5	2.7
$TiCl_4$	0	1.5	2.5
$ZrOCl_2$	0.7	2.0	3.0
$VOSO_4$	0	0	1.7
$NaVO_3$	0	0	0
$CrCl_3$	0.5	3.0	2.5
CrO_3	0	0.2	1.0
K_2CrO_4	0	0	0
$K_2Cr_2O_7$	0	0	0
Na_2MoO_4	0	0	0
Na_2WO_4	0	0	0
$MnCl_2$	0	0	0.2
$KMnO_4$	2.2	3.0	3.0
$FeCl_2$	0	3.0	2.2
$FeCl_3$	0	2.2	3.0
$FeSO_4$	1.2	3.0	2.2
*$Fe_2(SO_4)_3$	0	1.2	1.2
$Fe(NO_3)_3$	0	2.0	2.0
$FeSO_4(NH_4)_2SO_4$	1.0	3.0	2.7
$Fe(NH_4).(SO_4)_2$	0.2	1.7	3.0
$K_4Fe(CN)_6$	0	0	0
$K_3Fe(CN)_6$	0	0	0
OsO_4	0	0	0
$CoCl_2$	0	0	3.0
$NiCl_2$	0	0	0
$PdCl_2$	0	1.0	1.2
PtK_2Cl_6	0	0	3.0

TABLE I. (Continued)

Compound	Cutaneous Calcinosis (scale: 0-3)		
	0.2 mM/l.	2 mM/l.	20 mM/l.
$CuCl_2$	0	0	3.0
$CuSO_4$	0	0	3.0
$Cu(NO_3)_2$	0	0	1.7
$Cu(CH_3COO)_2$	0	0	2.0
*Ag_2SO_4	0	0.7	1.0
$AgNO_3$	0	0.5	1.2
$AgCH_3COO$	0	0.5	1.5
$ZnCl_2$	0	1.8	2.8
$CdCl_2$	0	1.8	3.0
$HgCl_2$	0	0.3	1.8
$Hg(CH_3COO)_2$	0	1.0	3.0
$AlCl_3$	0	1.2	1.0
*$Al_2(SO_4)_3$	0	1.2	0.7
$Al(NO_3)_3$	0.5	2.0	1.5
$Al(NH_4)(SO_4)_2$	0	1.2	2.2
$AlK(SO_4)_2$	1.0	1.0	2.0
$TlCl$	0	0	0
$TlCH_3COO$	0	0	0
$SnCl_2$	0	0.3	1.0
Na_2SnO_3	0.7	0.2	2.2
$PbCl_2$	0	2.0	3.0
$Pb(NO_3)_2$	0.2	2.0	3.0
$Pb(CH_3COO)_2$	0.2	2.0	3.0
$BiCl_3$	0	0.8	1.3
$Na_2B_4O_7$	0	0	0
Na_2CO_3	0	0	0
$NaHCO_3$	0	0	0
$NaNO_2$	0	0	0
NaH_2PO_4	0	0	0
$NaAsO_2$	0	0	0
Na_2HAsO_4	0	0	0
Na_2SO_3	0	0	0
$Na_2S_2O_3$	0	0	0
$KSCN$	0	0	0
Na_2SeO_3	0	0	0
$KClO_3$	0	0	0
$KClO_4$	0	0	0
KBr	0	0	0
KI	0	0	0
KIO_3	0	0	0
KIO_4	0	0	0.2

TABLE I cont'd (Footnote)

All groups were treated with DHT during the first 4 days, as indicated in the text. On the 4th day, 0.2 ml, of each aqueous solution was injected subcutaneously, at three different concentrations, into three different sites on the shaved back. The readings listed here represent the mean degree of calcification found on the 11th day.

* These compounds were injected at the concentrations of 0.1 mM/l., 1 mM/l. and 10 mM/l. instead of 0.2 mM/l,. 2 mM/l. and 20 mM/l., respectively.

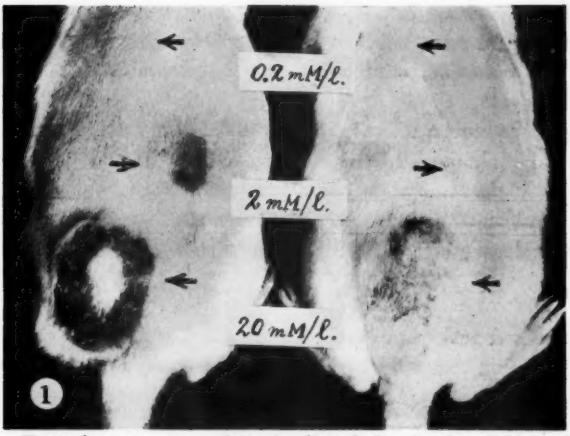

Fig. 1. Typical cutaneous calcinosis elicited by local injection of chemical compounds, on the back of DHT-pretreated rats. Photographed on 7th day after injection. Animal on left shows that skin in the center of calcified area produced by the highest concentration remains normal.

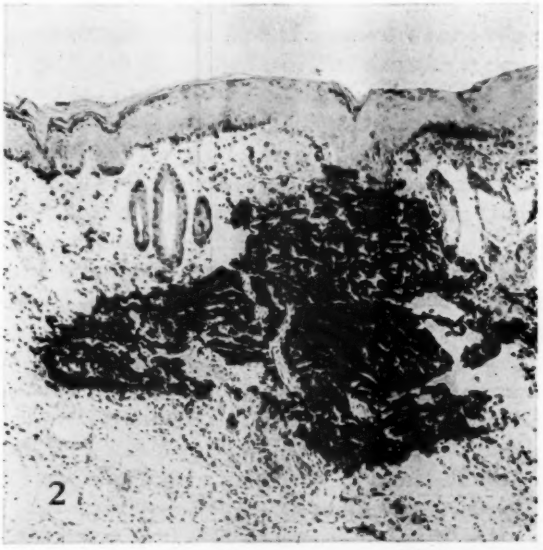

Fig. 2. Histologic appearance after subcutaneous injection with $FeCl_3$ (0.2 ml. of 2 mM/1. solution, given on 4th day, after DHT-pretreatment).

Spotty calcium deposition in subepithelial collagen fibers surrounded by inflammatory cells and some giant cells.

Note thickening of collagen fibers around mineral deposition.

(Alcohol-formol fixation, von Kóssa staining, × 100.)

The non-metal elements also, when given as their most common anions of sodium or potassium salts, have no calcifying activity, except $KClO_4$, which, at the highest concentration, produced a just detectable grade of calcification in only one of our rats.

Perusal of the results indicates that most metal salts are active. Iron, as a cation, invariably shows potent activity in eliciting lesions, as is illustrated in Figures 1 and 2. In some cases, the degree of calcification does not run parallel to the concentration of the solution given : necrosis caused by higher concentrations was sometimes accompanied by a low degree of calcification.

DISCUSSION

In rats treated with parathyroid hormone or DHT, cutaneous calcinosis with sclerosis is produced, not only by mechanical trauma, but also by the local injection of many chemical agents. We have tried various substances; however, at present it is difficult to say what kind of pharmacologic action is involved in the production of cutaneous calcification. For instance, as is seen in the table, some of the most active compounds, i.e., $KMnO_4$, $FeCl_3$, $CuSO_4$, $AgNO_3$, $Al_2(SO_4)_3$, $Pb(CH_3COO)_2$ and alums, have been used clinically as astringent, tannic or protein-coagulating agents. On the other hand, according to preliminary experiments, many organic compounds used as astringent, tannic or protein-coagulating agents (*e.g.*, tannic acid, picric acid, sulfosalicylic acid, trichloroacetic acid, formaldehyde ethanol and acetone) were not active at the same concentration when injected subcutaneously in the rats pretreated with DHT.

However, preliminary experiments also showed that croton oil, one of the strongest phlogistic agents, does not exert calcifying tendencies in the cutaneous tissue of the DHT-pretreated rat; on the other hand, local injection of triamcinolone, a strongly antiphlogistic corticoid, produces cutaneous calcinosis in similarly treated rats. It is already well known that, not only in experimental animals, but also in clinical cases, calcification of the soft tissue usually occurs when the acute phase of inflammation has ceased. Moreover, among the mechanical traumas, epilation is one of the most effective means of producing cutaneous calcinosis, but the lesion is best produced when epilation is performed at the "critical period," i.e., during the last 2 days of DHT-treatment.[6]

Thus, we know that the timing between the period of DHT-treatment and the phase of inflammation caused by mechanical or chemical trauma is an important factor in the production of experimental cutaneous calcinosis. Most chemical compounds when given subcutaneously cause a more or less local inflammatory raction; but if the optimal phase of inflammation, which varies with each element, does not coincide with the optimal phase of DHT-sensitization, calcification may fail to occur.

Because there was no significant difference among the anion groups of active

compounds, it can be said that specific characteristics of chemical elements play a decisive role in producing cutaneous calcinosis in rats treated with DHT.

SUMMARY

1. Eighty-six compounds were screened for their local activity in eliciting cutaneous calcinosis in rats pretreated with dihydrotachysterol.

2. Alkali and alkali earth metals, as well as non-metallic elements when given as their common inorganic anion of sodium or potassium salts, were not active in producing cutaneous calcinosis.

3. Most metal elements, except alkali and alkali earth metal elements were active; but their effect seemed to depend on the specific characteristics of the elements themsevles.

4. Iron as a cation was one of the most active elements in eliciting cutaneous calcinosis.

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Electrical Activity of Carp's Retina
Part 1. Graded Responses

By

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INTRODUCTION

It is generally accepted that the ERG of the vertebrate eye is a composite electrical potential consisting of three components; PI, PII, and PIII which were analyzed by Granit[1]. In his first monograph, Granit stated that PI was localized in the rod pathway, PII in the bipolar cell layer and PIII in the receptors and bipolar cell layer. Recently a problem on the origin of the ERG has been attacked by means of the microelectrode technique by Tomita and his associates[2-5], Ottoson and Svaetichin[6,7], Brindley[8,9] and others[10-13], but it is not entirely solved as yet.

Tomita and his associates have maintained that the b-wave (PII) and the a-wave (PIII) had their origin in the bipolar cell layer[2-5]. On the other hand, there are some evidences which lend support to the view that the ERG originates mainly in the receptors.[6,7,8,14,15]

The author has been investigating on the retinal action potential in the inverted preparation of the carp's retina with Motokawa and others. In the meantime, we reported that when the retina was illuminated by a small spot of light a positive sustained response was obtained in the illuminated field, while a negative response was recorded in the surrounding not intentionally illuminated field (the polarity of a response was relative to the diffuse electrode placed beneath the vitreous side of the retina). The positive response was inferred to be attributable to the activity of the receptors and the negative one to a more proximal layer, perhaps in the outer plexiform or the inner nuclear layer.[16,17] The positive response is homologous with the PIII component in polarity.

The carp's ERG recorded from an eye-cup preparation was found to be predominated by the component PIII, just like that of the Greek tortoise's REG which was described by Bernhard[19]. So the carp's eye will serve as an excellent material for study on this component.

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In the present paper ERGs of the toad and the carp were compared with special reference to the histological differences of both retinae, and it was emphasized that more than one kind of structure of the retina participate in the generation of the ERG.

METHOD

In these experiments Bufo vulgaris and Cyprinus carpio were used. After excision of an eye ball, the anterior part of the eye, cornea and lens, were cut away and the vitreous humor was removed by sucking with a small syringe as thoroughly as possible. The vitreous jelly of the carp's eye could often be picked up *en masse* by gentle suction. Sometimes such removal of the humor was not performed. Preparation was usually carried out in a dimly lighted room,but sometimes in a wholly light-adapted or dark-adapted state. The eye-cup preparation was mounted with the vitreous side upwards on a piece of Ringer-soaked black cloth which in turn was placed on the silver plate 1×1 cm. in size. This plate served as a diffuse electrode. A 3 M KCl-filled micropipette with D. C. resistance of about 20 MΩ was usually used for deep recording and a tungsten wire electrode coated with Insl X except at the tip (tip diameter about 1 μ) for surface recording.

The electrical phenomena were amplified with conventional R.C. coupled amplifiers with cathode follower input stages and displayed on a dual beam cathode-ray oscilloscope. The upper beam of the C. R. O. was used to display a surface response and the lower one for a deep response. Sometimes a low gain D. C. amplifier was used to check the changes of D. C. potential during the penetration of the microelectrode through the retina and also to records S-potentials. The output of the D. C. amplifier was fed into the upper beam. The overall time constant of the R. C. amplifiers used was 1.5 sec.

The optical arrangement used was the same as previously described[16,17], except a light source for stimulation which was a Sylvania glow modulator tube. The maximal intensity of the stimulating light measured 500 lux on the retina, when the maximal current of 70 mA was flowed through the tube. The duration of the stimulus was usually 0.9 sec. The size of the illumination on the retina was varied by means of a photographic stop placed in the light path. When background illumination was necessary, another light source was used. The intensity of the stimulating light was varied by means of neutral tint filters whose transmission factors were expressed as 0.7^n.

For analytical purpose, 1 % KCl and 10% ethylalcohol Ringer's solutions were applied to the retina by the aid of a small syringe.

Histological comparison was made on hematoxylin-eosin stained preparations.

The experiments were carried out at the room temperature ranging between

7° and 10°C in winter.

RESULTS

1. *Comparison of toad's and carps ERGs*

ERGs recorded from eye-cup preparations of both animals were compared, as shown in Fig. 1. The toad's ERG was usually characterized by well-marked a-, b- and d- waves. In contrast, the carp's ERG was predominated by a negative sustained potential which might remind us of the isolated PIII component of Granit's analysis of the vertebrate ERG. In the eye-cup preparation of the carp the b-wave was almost missing as long as the vitreous humor was not removed completely. In fact, it has been reported that negative ERGs such as those obtained from the carp's eye were obtained also in the frog's eye after massage[20,21] or treatment with KCl[22] or in exposure to low temperature[23,1], so it might be suspected that the negative ERG of the carp's eye would have been caused by these external agents during preparation. This is, however, not the case: The difference mentioned above was reproducible, when the preparations were made in one and the same procedure and with great care from both kinds of eyes.

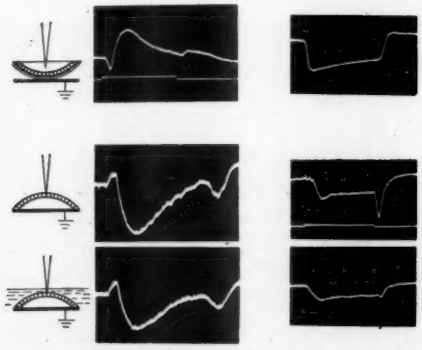

Fig. 1. Comparison of toad's and carp's ERGs. Position of recording and indifferent electrodes relative to preparation are shown on left. From top downward eye-cup preparation, inverted retina exposed in air and inverted retina perfused with Ringer's solution. Photographic records in left column were obtained from toad's eye and those in right column from carp's eye. In this and following Figs. upward deflection indicates increasing positivity of recording electrode against diffuse one. Light stimulus was even-illumination. Its duration and intensity were 0.9 sec. and 500 lux respectively.

Recently Gouras[24] has reported that a normal ERG of a bream eye *in situ* consisted of a-, b- and d-waves as in other vertebrate eyes, but in an excised eye a large negative ERG was frequently observed. He considered that this would be a sign of deterioration of the preparation. In the present experiment some of

carp's eye-cup preparations, showing a usual negative ERG, produced a positive ERG after the vitreous humor had been completely removed. Hence, it is apparent that deterioration is not the only factor to make the ERG negative. After recording of the ERGs from these eye-cup preparations they were in turn mounted on the black cloth with the vitreous side downwards, and the sclera and pigment epithelium were removed as gently as possible not to give any damage to the underlying retinal structure. These preparations are called an inverted preparation. Thus electrical responses were led off from the receptor layer with a microelectrode against the diffuse electrode placed on the vitreous side (see middle records in Fig. 1). In the following description the polarity of responses will be referred to the microelectrode. The response recorded from the toad's inverted retina was almost a mirror image of the normal ERG. In contrast, the response from the carp's inverted retina was not a mirror image, but negative as the normal ERG. A sharp off effect was to be noted.

In the previous papers[16,18] it was reported that in the carp's inverted retina a positive response was produced within a small illuminated area less than 1 mm. in diameter on the retina, while the response pattern was changed into a complicated negative form as the illuminated field was further increased. Such an area effect was interpreted as a result of interference between the standing positive potential generated in the receptor layer and the diffuse negative response generated in the layer by 100μ or so more proximal to the receptor layer. But this kind of area effect of illumination was not found in the toad's inverted retina, although Brindley reported that the response led off from the receptor surface of the frog's inverted retina changed its main polarity from negativity to positivity as the illuminated area was increased.

Next, these inverted preparations were immersed into Ringer's solutions. With the toad's retina the response remained almost the same as before after the immersion (see Fig. 1), while in the carp's retina the negative response was decreased to some extent. Especially the negative sharp off response was almost abolished. In other carp's preparations the positive responses evoked by a small spot of light centered upon the recording electrode placed at the receptor surface were promptly abolished by immersing the preparations into Ringer's solution. In these preparations large illumination produced large negative responses, which were also reduced to some extent on immersing the preparations into Ringer's solution. It is to be noted that the positive responses were much more liable to the shunting effect than the negative ones. A possible explanation of these findings would be that in the carp's inverted retina the positive responses occur in the receptor layer and that the negative responses which were more resistant to the shunting effect of the perfusing solution are located in the more proximal layer. On the other hand, the responses recorded from the toad's inverted retina would occur in the proximal layer to the receptors.

2. Depth recording

A recording micropipette electrode was lowered into the eye-cup preparation from its vitreous side, the inner surface of the retina being exposed in air by complete removal of the vitreous humor. The position of the electrode tip was measured by readings of a micrometer gauge attached to the micromanipulator. Results obtained in the toad's eye were almost the same as those of Tomita's experiments. As shown in Fig. 2, the normal ERG with small spike discharges

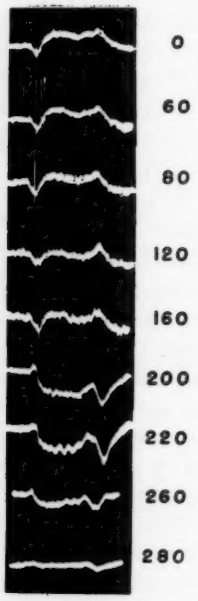

Fig. 2.

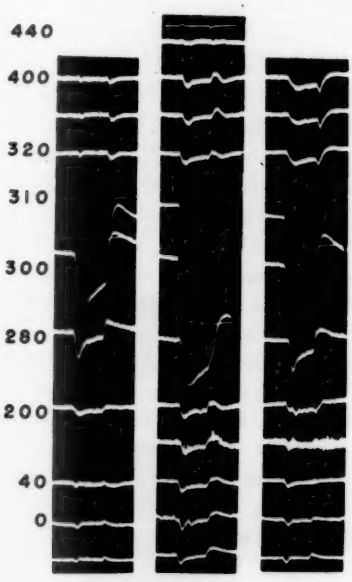

Fig. 3.

Fig. 2. Intraretinal recordings in toad's retina. A 3 M KCl-filled micropipette electrode was inserted from inner surface of retina which was exposed by complete removal of vitreous humor. Numerals on right indicate apparent depths of electrode tip from inner surface. Light stimulus was 500 lux in intensity and 0.9 sec. in duration. Apparent depth of 60μ would correspond to GCL in Fig. 8.

Fig. 3. Intraretinal recordings in carp's retina. Eye-cup preparation was used from which vitreous humor was not removed. Numerals show apparent depths of electrode tip from inner surface of retina. Lowermost records were obtained when electrode tip was still in vitreous humor. Thin traces represent records obtained with low gain D.C. amplifier. In these experiments three kinds of light stimuli were employed: Small spot of light 400×400μ in size (left column), even-illumination (middle column) and annular stimulus whose inner and outer diameters were 3mm. and 4mm. respectively (right column). Apparent depth of 120μ (fourth records from bottom) where spike discharge was recorded would correspond to ganglion cell layer (GCL) and apparent depth of 300μ to outer plexiform layer (OPL) or inner nuclear layer (INL) (cf. Fig. 9)

superimposed was recorded at the surface. At the depth of about 60–80μ, large positive spikes were recorded which means that the electrode tip arrived at the optic nerve fiber layer or retinal ganglion cell layer.

The ERG showed no remarkable change until the electrode tip reached a depth of 160μ. When the electrode was advanced to a depth of 200μ, a change in polarity took place concerning all the waves of the ERG. Oscillatory potentials were observed at further depths and at the depth of 280–300μ all responses disappeared. Histological examination of the toad's retina has revealed that the total thickness of the retina is about 250μ and that the inner nuclear layer or the bipolar layer is located at the distance of about 150μ from the inner surface of the retina. Thus it may be justified to suppose that the phase reversal of the ERG of the toad has occurred about in the bipolar cell layer.

Results obtained in the carp's eye-cup preparation are shown in Fig. 3. In this case three types of light stimuli were used: A small spot of light 400$\times$ 400μ in size on the retina (left column), a large circular illumination covering the whole preparation (middle column) and an annular stimulus of 3 mm. in inner diameter and 4 mm. in outer diameter (right column). It is to be noted that a small spot stimulus gave rise to a slightly positive response in contrast to the negative response to the large spot of light. When the electrode tip was lowered about 280μ from the inner surface of the retina, large negative responses appeared to the three kinds of stimuli, accompanied by a small amount of D.C. shift towards negativity. Advancement by further 20μ or so of the electrode gave rise to a great amount of a negative shift of the resting level (about -30 mV) and at the same time large negative responses to light stimuli were recorded which seemed to be S-potentials. After passage of this layer small responses similar to the surface responses in configuration were recorded again until their total elimination at the depth of 440 μ. In these series of experiments it was noted that the negative responses to the annular stimulus became larger at the deeper layer.

Furthermore, it should be emphasized that in the carp's eye-cup preparation there was not observed the reversal of the ERG, which was usually observed in the toad's eye-cup preparation. When the microelectrode was a little grosser, e.g., 1–2μ in tip diameter, it was often observed that almost constant negative responses were recorded all the way from the inner surface to the depth of as far as 1 mm. from the surface, although the S-potential was encountered at the depth of about 300μ from the inner surface as usual. This finding suggests that the gross electrode could not penetrate but was pressing a certain tough membrane lining behind the layer where the S-potential originated. This membrane may be the external limiting membrane or Bruch's membrane.

The experiments of the inverted retina of the carp showed that even a gross microelectrode of 10μ in tip diameter could get through the whole retina very

easily : At the depth of 350-400μ from the receptor surface all the responses had disappeared. Consequently the relevant resistant membrane of the eye-cup preparation would be supposed to be Bruch's membrane.

3. Effects of KCl and ethylalcohol upon the intraretinal responses and ERGs

It has been reported that KCl selectively suppresses the positive component PII and that ethylalcohol selectively suppresses the negative component PIII.

So effects of 1% KCl and 10% ethylalcohol solutions upon the intraretinal action potentials and the ERGs were studied in the eye-cup preparations from which the vitreous humor was completely removed. For the purpose of examining the relation between the intraretinal responses and the surface ERGs a tungsten wire electrode was placed on the vitreous surface of the retina and a

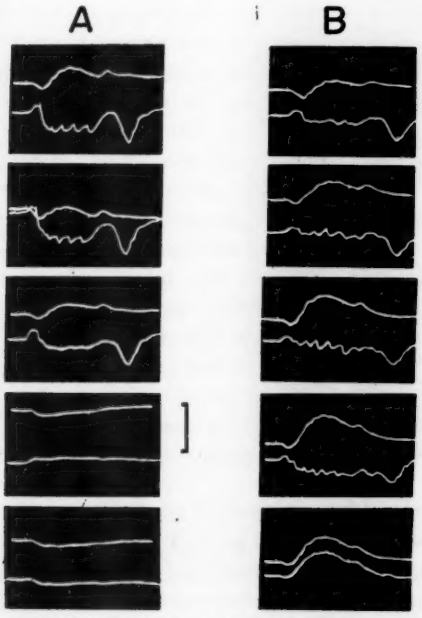

Fig. 4.A. Effects of 1% KCl solution upon ERG and intraretinal response of toad's eye. In this and following Figs. upper and lower records in each pair show ERG and deep response respectively. In this case deep electrode was fixed at depth of 200 μ from inner surface of retina. Records, from top downward, were taken before, immediately after, 1 min. and 5 min. after application. Bottom record was taken on withdrawal of electrode by 220μ 6 min. after application.

B. Effects of 10% ethylalcohol. Deep electrode was fixed at depth of 180 μ. Records, from top downward, show control, immediately after, 2 min. and 5 min. after application. Bottom record was taken on withdrawal of electrode by 200 μ. Calibration 500 μV.

micropipette electrode was fixed at an appropriate depth in the retina.

In the toad's retina the deep electrode was placed at the depth of about 200μ. As can be seen in Fig. 4A, the surface electrode recorded the normal ERG which was illustrated in the upper record of the top photograph, while the deep electrode picked up the negative intraretinal response characterized by marked oscillatory potentials (the lower record). When 1% KCl solution was applied to the retina, changes in both the surface and deep responses occurred. First, the oscillatory potentials were abolished and the b-wave and its counterpart in the deep response were reduced. As this stage apparently the a-wave together with its counterpart and the off-effect were not affected yet (see the third record from top). 5 minutes after application of KCl, all the other waves except the a-wave were obliterated. Consequently there remained only a negative monophasic potential in the surface lead and its counterpart, a positive monophasic potential in the deep lead. At this time the deep electrode was withdrawn by about 60μ, where no response was picked up. When the electrode was withdrawn further by 160μ, the same response as at the surface was obtained (bottom record in Fig. 4A).

As far as the surface response is concerned, the effects of 1% KCl solution were similar to what was described by Therman and others.

Next, the action of 10% ethylalcohol solution was tested in other preparations. Fig. 4B shows a series of records obtained in these experiments. The deep electrode was situated at the depth of about 180μ. When the alcohol was applied to the surface of the retina, the oscillatory potentials were soon exaggerated. With the lapse of time the oscillatory potentials grew more marked and appeared even on the off response. The a-wave and its counterpart were reduced while the b-wave and its counterpart were augmented.

Fig. 5 shows the experiments on the carp's eye. In these cases the deep electrode was fixed more distal to the layer where the S-potential was picked up. The effects of 1% KCl solution are seen in Fig. 5A. About 2 minutes after the application of this chemical the deep response turned from a little complicated negative potential to a simple monophasic positive response.

When deprived of their vitreous humor, retinae of the carp's eye-cup preparations sometimes showed variable electrical activities. When they were prepared under very bright light-adaptation, positive responses of complicated shape were often obtained from the inner surface as shown in the top record of Fig. 5B. At this time deep responses were positive or negative as the case might be. Such anomalous responses tended to become normal while the preparations were kept in a dark room. Changes in size and intensity of the stimulus light also elicited such anomalous phenomena: A large-sized illumination evoked a normal negative surface response, but a small-sized one produced positive responses; strong illumination caused negative responses, while weak one positive responses. One of examples which showed an alteration of polarity of a response

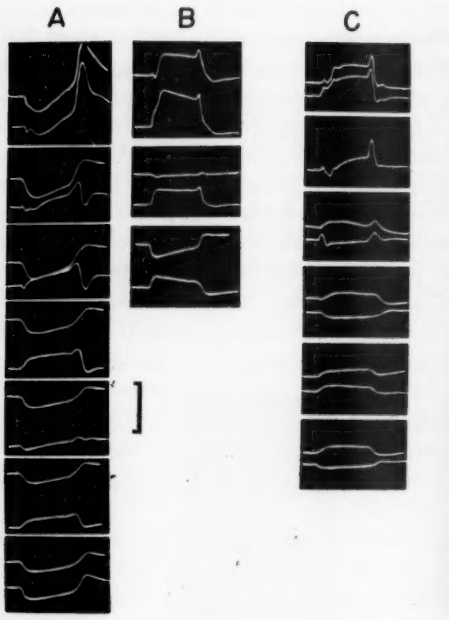

Fig. 5.

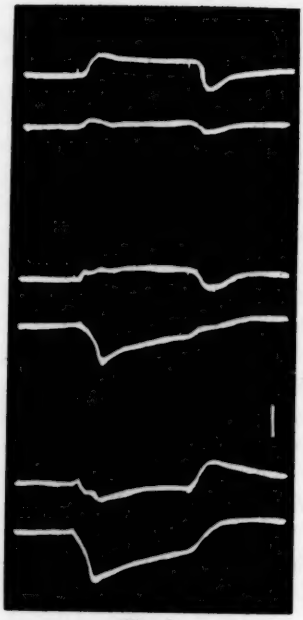

Fig. 6.

Fig. 5. A. Effects of 1% KCl solution upon ERG and deep response of carp's retina. Deep electrode was fixed at depth of 300 μ from inner surface of retina. Records, from top downward, control, 30 sec., 1 min., 2 min. after application. Lower three records were taken as follows ; 5 min. after application deep electrode was withdrawn by 100 μ and again advanced by 100 μ and then withdrawn by 400 μ.

B. Effects of 1% KCl solution upon anomalous positive ERG and deep response. Deep electrode was fixed at depth of 350 μ. From top downward, control, 2 min. and 5 min. after application.

C. Effects of 10% ethylalcohol upon ERG and deep response of carp's retina. Deep electrode was fixed at depth of 350 μ. From top downward, control, 30 sec., 1 min., 2 min. after application. Lower two records were taken at depths of 0 and 300 μ 5 min. after application. Calibration 500 μV.

Fig. 6. Changes in patterns of ERG and intraretinal responses depending upon intensity level. Intensities were 500, 60 and 15 lux from bottom upward. Vertical bar shows calibration : 500 μ V for upper beam and 2 mV for lower trace.

depending upon the intensity of stimulus is illustrated in Fig. 6. The lower record in each pair represents responses obtained at the depth of about 200 μ. When weak light fell upon the large field of the retina, both recordings showed positive responses (top recordings), but on a little stronger illumination the deep response indicated negativity in contrast to the still positive surface response (middle recordings). Illumination of maximal intensity made both recordings

negative (bottom recordings). Such different behavior of the surface and deep responses suggests that the deep and surface responses do not reflect the same electrical activities.

Effects of KCl upon the anomalous positive surface responses were tested, as shown in Fig. 5B. The surface response changed its polarity from positive to negative, but the deep response showed no polarity change, remaining positive as seen in Fig. 5A. Thus KCl exerted similar effects upon the responses of the carp's retina whether positive or negative. Fig. 5C illustrates some records taken in experiments on the effects of 10% ethylalcohol. Before application of alcohol, both the surface and deep responses showed positive patterns. After application the surface response gradually changed into a simple positive configuration, while the deep response became simply negative.

4. Dependence of S-potentials upon the intensities of stimulating lights

The S-potential has been subjected to many investigations since the first description of it by Svaetichin[26]. It has universally been accepted that the S-potential originates in the outer plexiform layer and inner nuclear layer of the vertbrate retina[27-30]. Three kinds of S-potentials have been reported in fish retina : L-type, Y-B type and R-G type. The latter two types show the polarity reversal depending upon the wave-lengths of stimulating light.

Recently Gouras[24] indicated that in the bream retina some S-potentials varied their polarity with changing intensities of the stimulating light. In the

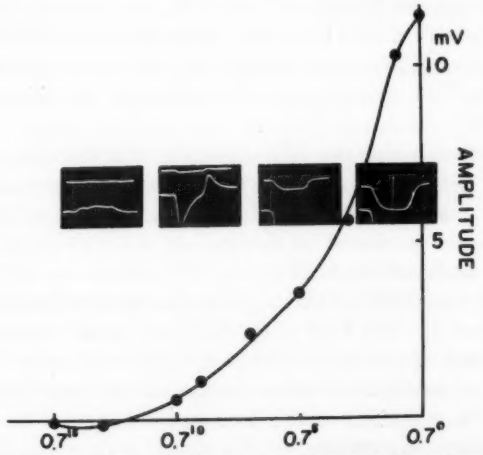

Fig. 7. Amplitudes of S-potentials are plotted against log. intensities of stimulating lights as abscissae. Upper and lower records in insets were taken with low gain D. C. amplifier and high gain R. C. amplifier. Unit of intensity is 500 lux. Note phase reversal at 0.7^{12}.

carp's retina such types of S-potentials were often observed, too. The intensity level of illumination where the polarity reversal of S-potentials occurred varied from case to case. One of examples is shown in Fig. 7 where response amplitude is plotted against log. intensity as abscissa, the unit of intensity being 500 lux. In this experiment it may be necessary to define temporarily a S-potential as a large graded response over 10 mV in amplitude for the maximal intensity of stimulating light, accompanied by D.C. shift more than 30 mV. Because the amplitude of the graded response was diverse and there seemed to be little correlation between amplitude of the graded response and D.C. shift, as reported by Tasaki in the common gold fish retina[31].

Recordings were made on two beams of the C.R.O. as described in methods to detect even small responses at lower levels of illumination. In the insets the polarity reversal is clearly shown, especially at a very low intensity level. Some S-potential did not show any trace of polarity reversal at all. No systematic investigations were performed to correlate such a change in polarity due to a change in intensity to three types of S-potentials mentioned above.

DISCUSSION

The normal ERG recorded from the carp's excised opened eye was found to be very much different from that of the frog's or toad's eye. The latter consisted of well-marked three waves a, b and d. But the carp's ERG was predominated by a large negative wave which seemed to represent the PIII as analyzed by Granit in the vertebrate ERG.

Bernhard[19] observed an ERG purely consisting of the PIII in the tortoise's eye whose retina is said to be a practically pure cone retina and suggested a close connection of the PIII with cone activity. However, he did not mention the origin of the PIII.

From experiments on the frog's eye, Tomita and his colleagues have maintained that the ERG originates in the bipolar cell layer on the basis of the fact that while advancing an microelectrode from the inner surface of the retina the ERG was found to reverse its direction in this very layer, suggesting the existence of an electrical dipole there.

With depth recordings in the present experiments I have confirmed the Tomita's experiments: The ERG of the toad's eye made a phase reversal in the vicinity of the bipolar cell layer. When the recordings were made from the receptor surface of the toad's inverted retina, a mirror image of the normal ERG was obtained. These findings would demonstrate the presence of an electrical dipole layer in the toad's retina.

In contrast, no phase reversal of the ERG took place when depth recordings were carried out in the carp's eye-cup preparation. The response obtained from the carp's inverted retina was not a mirror image of the normal ERG. These

experimental findings can not be explained from a simple assumption of a single electrical dipole layer in the carp's retina. The question will not be solved from the above experiments as to whether there is no dipole layer in the carp's retina or whether there are many dipole layers of different polarities lying so closely to one another that it is difficult to detect them.

When 1% KCl solution was applied to the inner surface of the carp's retina, the ERG showed the pure negative PIII and the corresponding deep response was a mirror image of it (see Figs. 5A and B). It seems that KCl uncovered an electrical dipole layer probably because the other dipole layers were destroyed by this agent.

Furthermore, ethylalcohol applied to the inner surface of the carp's retina has revealed the presence of another kind of dipole layer; the changed ERG consisted of a pure positive phase corresponding to the PII and the counterpart of the deep recording was a pure negative potential (see Fig. 5C). From these analyses it has been elucidated that there are at least two dipole layers of different polarities in the carp's retina.

It will not suffice to assume only one layer for generation of the carp's ERG, but at least two kinds of layer, e.g., the receptor layer including the outer plexiform layer and the bipolar cell layer will emerge as probable seats for the two dipole layers of different polarities.

The vertebrate retina is roughly divided into three layers, i.e., the receptor layer, the bipolar cell layer and the retinal ganglion cell layer. The innermost layer, the ganglion cell layer, together with the optic nerve fiber layer discharges only spikes which follow the all-or-none law.

According to Granit and Helme,[32] antidromic discharge of the ganglion cell neither altered nor evoked the ERG. Furthermore, it was reported that inactivation of the neural elements with cocaine (Ottoson and Svaetichin[6]) and the degeneration of them after dissection of the optic nerve (Noell[15]) brought about no essential change in the ERG. From these findings it is not likely that the electrical activities in the ganglion cell layer contribute to the generation of the ERG.

In addition, the remarkable differences in the configuration of both ERGs of toad's and carp's eyes could be explained in view of the histological differences of both retinae.

Figs. 8 and 9 show the histological pictures of toad's and carp's retinae, respectively. It is clear that the outer nuclear layer is thinner and containing a smaller number of constituent nuclei in the toad's retina than in the carp's one, while the inner nuclear layer is thicker and occupied by more nuclei in the toad's retina than in the carp's one. In the inner nuclear layer of the carp's retina it is to be noted that there are some brightly stained large cells here and there.

In the carp's retina in which the outer nuclear layer predominates, the

Fig. 8. Microphotograph of toad's retina. Labelling : BL, bacillary layer ; ONL, outer nuclear layer : OPL, outer plexiform layer ; INL, inner nuclear layer ; LPI, inner plexiform layer ; GCL, ganglion cell layer ; OPT, layer of optic nerve fibers. Vertical bar indicates 200μ in the retina.

Fig. 9. Microphotograph of carp's retina. Labelling ; the same as in Fig. 8. Vertical bar indicates 280μ in retina.

negative component PIII of the REG is well developed. In the toad's retina in which the innernuclear layer predominates the positive component PII prevails. The simplest interpretation of these facts would be that the PIII is produced in the outer nuclear layer while the PII is generated in the inner nuclear layer.

It has been shown in the experiment with inverted carp's retina that the positive potential which may be regarded as corresponding to the PIII is more liable to the shunting effect than the negative one corresponding to the PII. In these preparations the outer nuclear layer is so near the surface of the preparation that the potential produced in this layer would more easily be shunted by immersion of the preparation into Ringer's solution. The above-mentioned interpretation seems to receive further support from the experimental fact previously reported[33] that electrical stimulation of the bipolar cell layer of the carp's retina with microelectrode inserted into the layer always produced a negative slow potential which coincided with the PII in polarity.

There is no essential difference between ERGs of the toad and the carp, although the differences in apparent patterns are striking as is shown in Fig. 1. The patterns of the ERG will be of the frog (or toad) type or carp type depending upon which of these two layers is more active.

According to Autrum's analytical experiments on the insect ERGs[34], the pattern of the ERG is dependent upon the degree of interference between the electrical activities of the receptors and those of more centrally located optic ganglions. Similarly the various patterns of the vertebrate ERGs would be dependent upon the degree of the electrical interaction between these two layers. As illustrated in Figs. 8 and 9 the two layers in question are situated more closely to each other in the toad than in the carp. Therefore, a stronger interference between both layers may be expected in the toad's retina. In fact, it is difficult to separate negative and positive components of ERG in the toad's inverted retina preparation, but it is usually easier in the carp's one[16,17]. Such closely situated dipole layers as found in the toad's retina can be represented by one dipole layer, so that the clear phase reversal could occur during advancement of the microelectrode through the retina. If two dipole layers of opposite polarities are separated to some extent, it is to be expected theoretically that two phase reversals of opposite directions would take place successively. In reality, however, our present technique would be insufficient to detect such a fine detail of the electric field. It must be the reason why in the carp's retina the phase reversal was not encountered.

The potentials in the two layers do not always change in parallel when external conditions are changed. Consequently not only the magnitude of the ERG. but also the pattern itself may change when the external conditions are changed. For example, as shown in Fig. 6, the ERG from the inner surface (upper record in each pair) turned from positive to negative as the intensity of illumination was increased. Similar changes could be observed with increasing areas of illumination.

In the above discussion, the intraretinally recorded potentials were referred to for the interpretation of the ERG, but all the intraretinal potentials do not

contribute to the ERG. The degree of contribution of the intraretinal potentials depends on the extent of their spread. So the extremely localized potentials such as S-potentials do not participate in the ERG.

SUMMARY

The ERGs of the toad and the carp were compared with special reference to the histological differences of both retinae.

1. A usual ERG of the carp was predominated by the negative PIII, while that of the toad consisted of well-marked, a, b and d-waves. It was to be noted that electrical responses recorded from the receptor surface of the carp's inverted retina was not a mirror image of the usual ERG, while those from the toad's inverted retina was a mirror image of the usual ERG.

2. Intraretinal recordings in the eye-cup preparations with a 3M KCl-filled micropipette electrode showed that in the toad's retina a phase reversal of all components of the ERG occurred at the depth of 160μ, but that in the carp's retina no phase reversal took place at any depth. From this finding it may seem that in the carp's retina there is no electrical dipole layer in contrast to the presence of it in the toad's retina.

3. When 1% KCl solution was applied to the inner surface of the retinae, the negative PIII component and its counterpart of opposite polarity in the intraretinal response were isolated in both retinae of the carp and the toad.

4. By application of 10% ethylalcohol solution to the inner surface of the retinae the positive component PII and its counterpart of negative polarity in the intraretinal response were isolated in the carp's retina. In toad's retina the action of this agent was to suppress the negative component of the ERG and its counterpart of positive polarity in the deep lead, and to augment the positive component of the ERG and its negative counterpart in the deep response.

5. The findings of 3 and 4 would show that there are at least two electrical dipole layers of opposite polarities in the carp's retina.

6. Histological examination of both retinae showed that the outer nuclear layer is thinner and containing a smaller number of constituent nuclei in the toad's retina than in the carp's one, while the inner nuclear layer is thicker and occupied by more nuclei in the toad's retina than in the carp's one.

7. From these findings the mechanism was discussed about the generation of the ERG.

8. Some S-potentials were found to show a phase reversal depending upon the intensity level of stimulating light.

The author is greatly indebted to Prof. K. Motokawa for his invaluable discussion in preparing this paper.

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EEG Analysis with Decatron Toposcope

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Recently the toposcopic procedure by Petsche (1954)[2] has added a unique device to electroencephalographic analysis. Most of the multi-channel EEG recordings which have been generally used for clinical and experimental purposes depend on ink-writing, so that the speed of recording is limited to a considerable extent.

On the other hand, although the cathode ray oscilloscope is generally fit for recording of rapid-moving phenomena, the use of large number of cathode ray tubes (CRT) is usually required for recording of a variety of phenomena. In order to improve these condition, Petsche constructed his first toposcope "Photozellen Toposcop"(1954)[3]. However, this apparatus had defects in ability of resolution and in its mechanism using flash light. Later, he presented a report on his experiments with a multivibrator toposcope[4], and finally succeeded in completing the toposcope (1960)[6].

We contemplated to construct a toposcope for our own EEG analysis and neurophysiological researches. As a result, we adopted a modified device with the purpose of removing a complicated circuit, making the construction easier and increasing the steady action and ability.

MECHANISM AND TECHNIQUE

The decatron toposcope in this paper is in possession of one decatron, as electric switch, instead of numerous multivibrators.

The repeated drive of Y axis (vertical) of CRT by the series of square pulses whose amplitude is stepwise decreased makes it possible to record multi-channel EEG on the single beam of CRT (Fig. 1). These pulses are readily induced from the out-put of the decatron (Figs. 1 and 2).

The out-put from CRT terminal of EEG apparatus is led to the grid of amplifiers corresponding to the respective channels. However, as these vacuum tubes are kept inactive by the attached switch tubes, the EEG signal is not delivered to the anode plate. The cathode of the amplifier tube is maintained

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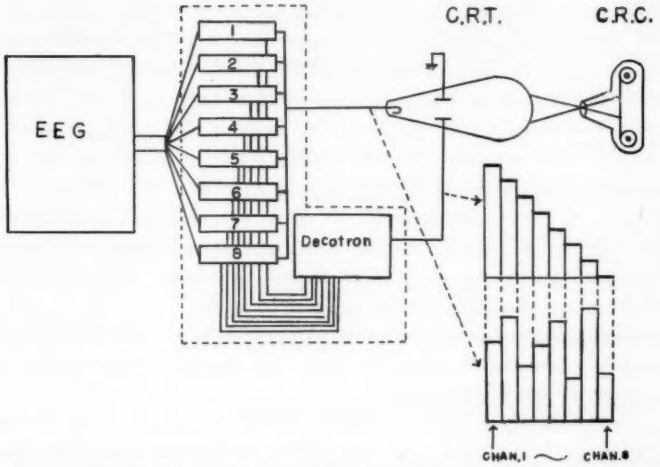

Fig. 1. Block diagram of the whole system and schematic signals into the vertical in-put (upper, stepwise square pulses) and the brightness modulator in-put (lower, EEG signals of each channel) of the cathode ray tube (C.R.T.). The single decatron generates all square pulses.
Recorded with the continuous recording camera (C.R.C.).

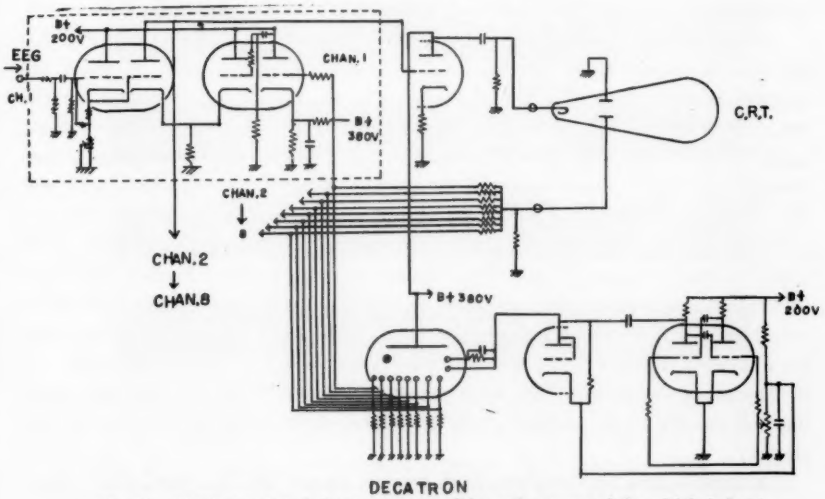

Fig. 2. EEG signal is led into the amplifier tube (upper left) which is kept inactive by the switch tube (right). When the switch pulse from the decatron is induced into the switch tube, the amplifier tube produces the out-put signal of EEG that modulates brightness of C.R.T., and that appears on the top of the corresponding vertical square pulse.
Each channel EEG appears successively with varying brightness.

in high voltage by the cathode of the switch tubes. However, as long as square pulses of the first step enter the switch tubes, the amplifier tubes become active whereby momentarily converting the EEG signal into the out-put. In the meanwhile, the remaining 7 amplifier tubes are inactive. As such an operation takes place successively, the amplitude of EEG of 1–8 leads are shown in sequence in the out-put common to 8 amplifiers though it lasts for a short duration. These signals modulate the brightness on Z axis of CRT, the brightness intensity corresponding to the amplitude variation of the first channel of EEG is shown on the top of the square pulse of the first step, and those of the other channels are similarly shown on the top of their corresponding pulses. By the repetition of these bright spots, there is seen the shading of light.

The frequency of square pulses in our apparatus is 2,400–40,000 cycles/sec. (maximum frequency of decatron : 40 KC), and the signal frequency in the case of 8 channels is 300–5,000 cycles/sec.

As mentioned above, EEG of 8 channels can be recorded with the variation of brightness on 8 lines as in the case of the ink-writing electroencephalogram. A continuous recording camera was used for recording.

PRACTICE

Citing examples, the author wishes to describe briefly the data acquired by us from the obtained electrotopogram (there is a detailed report by Petsche[2,4]).

In this paper the bipolar leading routine EEG of alpha rhythm (Fig. 3a) and its topogram (Fig. 3b) are shown. A band consists of 8 lines, in which the electroencephalograms of 8 leads are shown in downward succession as the variation of brightness. The bright part of each line indicates the positive phase to the base line of alpha waves, the shadow part of it being the negative phase. Accordingly, the brightest part and the darkest part show the positive peak and the negative peak of each alpha wave, respectively. What is of greatest importance is the correlation between brightness and shadow of these 8 lines. The upper 4 leads belong to the left hemisphere and lower 4 leads to the right hemisphere, between which there is seen phase shifting with the occasional occurrence of 180° reversal change (Fig. 3b). Phases of the 4th and 8th leads are obviously different from those of other leads, thus being tempolar leads. As in the case of the routine EEG, alpha blocking phenomena are shown by eye opening and the variation of brightness by alpha waves is restored again by eye closing.

Stripes caused by the phase shift become clearer in the monopolar leading (Fig. 4a) and the topogram (Fig. 4b) in 7 electrodes placed from the nasion to inion.

It is demonstrated that most of these stripes have some gradient; there are the successive inclination of the alpha wave phase from the nasion to the inion

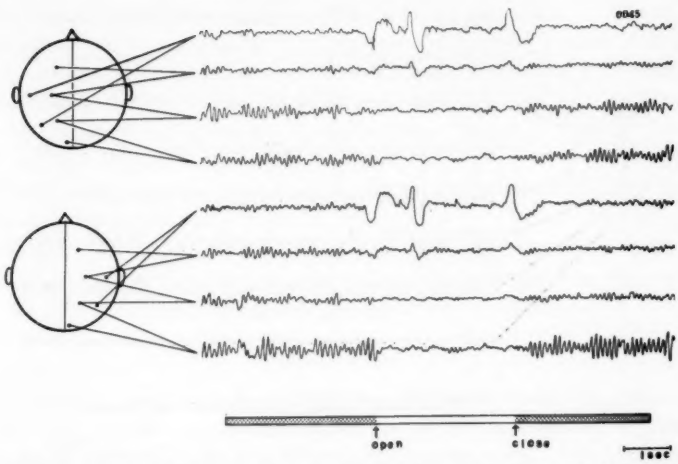

Fig. 3a. Bipolar leading routine EEG, including alpha wave blocking by eye opening. Recorded simultaneously with topogram (see Fig. 3b).

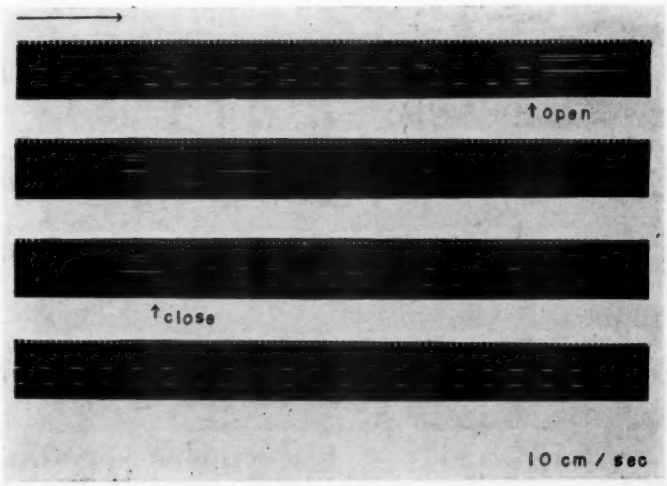

Fig. 3b. A continuous recording of topogram. Each band consisting of 8 lines shows 1-8 channel EEG in downward succession. The upper edge indicates 20 msec. timer. Film speed : 10 cm/sec.

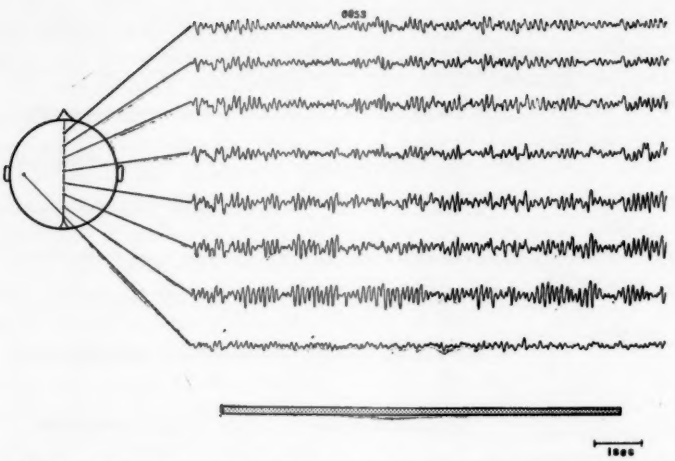

Fig. 4a. Monopolar leading EEG of normal alpha waves. Electrodes are placed in alignment from nasion to inion except one on the left temporal area. Simultaneous recording with the topogram (Fig. 4b).

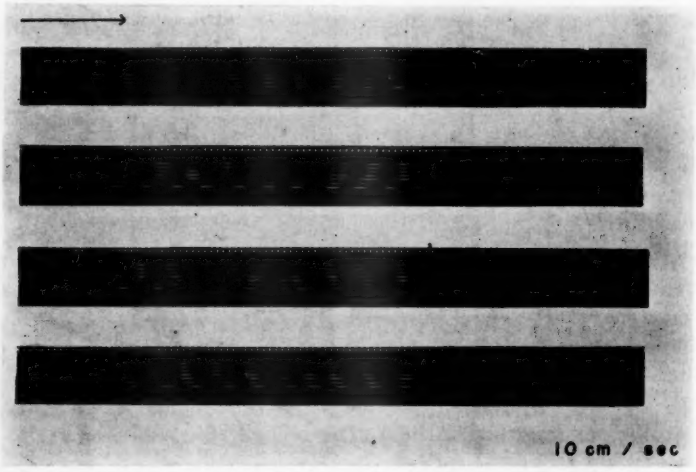

Fig. 4b. Dark and bright stripes and their vertical gradients. Timer : 20 msec. Film speed : 10cm/sec.

and the reversal transference of it from the inion to the nasion.

Granting it to be a spreading in appearance, this would provide a basis for the assertion that alpha waves are spread to a certain direction. It is interesting that the direction of this spreading is reversed with the lapse of time (Fig. 4b). Moreover, the reversal of direction is seen 3–4 times within 10 seconds. In this respect, we agree with the finding by Petsche[2].

Although these facts are not known simply by the routine EEG, they become obvious with the toposcope.

Moreover, not only these notable facts but also the phase shifting is measurable. If the distance between the leading electrodes and the film speed is given, the speed of spreading can be measured without difficulty.

DISCUSSION

The decatron toposcope is a polyscope fitted and simplified for our experimental and clinical purposes. This toposcope enables us to observe phenomena of biological electric activity simultaneously and at desirable speed. This observation includes not only the phase correlations among wave forms of multi-channel leading EEG which are hardly observable generally but also the spreading pattern, direction, speed, etc. However, as the decatron toposcope depends on phenomena by brightness modulation, we should pay attention to the fact that the ability of regeneration of oscilloscope screen and the gradation of photo-film or -paper are concerned in the data. Accordingly, the decatron toposcope is not fit for the measurement of wave form and voltage.

The adequate signal frequency for our observation of EEG was 800 cycles/sec., but in case of film speed (10 cm/sec) adapted for that of alpha wave the 8th lead showed always a delay of 10/800 cm for that of the first lead. However, this delay was negligible as compared with other factors.

Prior to recording, it is necessary to adjust the base line to light brightness so that the bottom to peak out-put voltage of the object phenomena can render pertinent shading.

SUMMARY

We designed a decatron toposcope which is simple in structure and easily produced. The result of our practical tests with the apparatus was the same as that by Petsche's multivibrator.

The use of this apparatus indicates to be useful for researches of EEG and neurophysiology.

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Study of Latent Carcinoma of the Prostate in the Japanese Based on Necropsy Material

By

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INTRODUCTION

Carcinoma of the prostate was recognized already in 1817 by Langstaff, but the first definite description should be attributed to Thompson, who collected 18 cases of prostatic cancer in his textbook published in 1857.

In the nineteenth century, prostatic carcinoma was thought to be a very rare disease, but in 1900, Albarran and Hallé[2] stated that they could find "epithelioma adenoide" in 14% of cases after histological examination of 100 surgical specimens of benign prostatic enlargement, and considered it as an intermediate form between ordinary senile hypertrophy and true prostatic carcinoma.

In 1926, Neller and Neubürger[43] examined 40 prostates of autopsy cases histologically, and found "atypische Epithelwucherung" in 7 cases, all of which showed various stages of transition to "echte metastasierende Karzinome", and concluded that these changes must be considered as "beginnende Karzinome" from the histological point of view.

Since then, many authors have reported similar observations of surgical specimens or of autopsy cases. The principal results of them are summarized in Table I. As indicated in Table I, these reporters coincide in the fact that even if there is no clinical sign of prostatic carcinoma, malignant foci can often be detected if examined histologically, and the incidence reaches up to 20% or more over the age of 50. At present, these opinions have gained general acceptance.

These malignant foci have been called by various names, such as "atypische Epithelwucherung", "beginnende Karzinome" (Neller u. Neubürger)[43], "histologische Karzinome" (Stehr)[51], "small carcinoma" (Moore)[38], "localized carcinoma" (Kahler)[32], "occult carcinoma" (Rich),[47] "latent carcinoma" (Andrews)[5], etc. Presently it is usually called by the name of latent carcinoma.

In our country, similar observations have been reported by Yotsuyanagi,[55]

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TABLE I. Incidence of Latent Prostatic Carcinoma in Literature

Authors	Nomenclature	Material	Incidence (%)
Neller u. Neubürger (1926)	atypische Epithelwucherungen und beginnende Karzinome	autopsy: 30-70 years old 40 cases	17.5
Mintz & Smith (1934)	early carcinoma	autopsy: 100	13
Rich (1935)	occult carcinoma	autopsy: over 50 292	14
Moore (1935)	small carcinoma	autopsy: 20-90 375	16.7
Bibus (1937)	carcinoma	operation: 430	4.5
Walthard (1937)	Karzinome	autopsy: over 40 100	30
Kahler (1939)	carcinoma	autopsy: over 50 123	17.3
Baron & Angrist (1941)	occult carcinoma	autopsy: 1) 364 over 50 2) 50	14.8 46
Oswald (1941)	sichere Karzinome	autopsy: over 40 120	12.5
Meyenburg & Cathomas (1948)	occult carcinoma	autopsy: over 40 100	23
Andrews (1949)	latent carcinoma	autopsy: over 40 142	12
Horstman (1952)	occult carcinoma	autopsy: 40-86 118	10.2
Allemann (1952)		operation: 280	5
Edwards *et al.* (1953)	latent carcinoma	autopsy: over 40 173	16.7
Totten *et al.* (1953)	latent carcinoma	operation: 116	20
Frank (1954)	latent carcinoma	autopsy: over 50 210	37.6

Abe,[1] Ichikawa *et al.*,[29] Ito[31] and Ohta *et al.*[44] These results are shown in Table II. According to Ohta *et al.*[44] latent carcinoma of the prostate can be found in our country as frequently as in Europe and the United States.

TABLE II. Incidence of Latent Prostatic Cancer in our Country

Authors	Nomenclature	Material	Incidence (%)
Yotsuyanagi (1938)	beginnende Karzinome	autopsy: 100 cases	3
Abe (1943)		autopsy: 550	1.8
Ichikawa *et al.* (1951)	latent carcinoma	operation: 43	5
Ito (1954)	latent carcinoma	operation: 101	5
Ohta *et al.* (1958)	latent carcinoma	autopsy: over 45 years old 203	13.3

As is well known, the mortality due to prostatic carcinoma in Japan is much less frequent than in Europe and the United States both clinically and pathologic-anatomically. According to Table III, showing the mortality due to prostatic carcinoma, based on the statistics by Segi *et al.*[48], there is a great discrepancy between the mortality due to the disease in Japan and that of the same disease in foreign countries. Therefore, the reports stating that the incidence of latent carcinoma of the prostate in our country is almost as frequent as in other countries, seem to be contradictory.

The purpose of this paper is to investigate the incidence of latent carcinoma

TABLE III. Corrected Death-rate of Prostatic Carcinoma in Various Countries (per 100, 000). (by Segi *et al.*)

Country	Death-rate	Country	Death-rate
America (coloured)	21.7	Holland	12.6
Norway	15.6	Scottland	12.5
Switzland	15.2	West Germany	11.9
Australia	15.1	England	11.8
Sweden	14.4	Finland	10.2
America (white)	14.0	Portugal	8.5
France	13.2	Italy	7.7
Austlia	13.1	Israel	7.3
Canada	12.8	Japan	1.1
Belgium	12.8		

of the prostate in autopsy cases in our country, and to study its histogenesis, the mode of invasion and others.

MATERIALS AND METHODS

Material used in this study is the prostate of 189 autopsy cases over the age of 40 dissected at the Pathological Department of Tohoku University from 1956 to 1959, and 40 autopsy cases over the age of 50 dissected at the Pathological Department of Fukushima Medical College from 1954 to 1958, that is, 229 prostates in total. Although three cases (No. 7'57, No. 187'57, and No. 149'59) which were diagnosed as prostatic carcinoma, clinically or on the autopsy table, and the cases incompletely preserved are not included in this series, no other selection was made except the age. Ten per cent solution of formalin was employed for fixation.

Methods: After dissecting the prostate from its surrounding tissues, the entire prostate was cut perpendicularly to the urethra, dividing it into pieces of tissue approximately 4 to 5 mm thick (step section method). After giving a number to each piece, and making a sketch of its macroscopic findings, they were embedded in paraffin, and histological preparations were made from each block. Thus, eight preparations in average and at least three preparations were obtained from single prostate. In this way, all the lesions, 1 cm or more in diameter, could be examined microscopically. H-E staining was employed routinely, but in cases of carcinoma or of carcinoma-like lesion, elastica-staining, Masson-Goldner's staining, Gomori's silver-impregnation, PAS-staining and mucicarmine-staining were added. If necessary, the serial section was made to pursue its extension, invasiveness and others.

CRITERIA OF MALIGNANCY AND GENERAL HISTOLOGICAL FEATURE

One of the reasons of great difference in the incidence of latent carcinoma

according to reporters seems to lie in the difference of criteria of malignancy adopted.

As criteria of "small carcinoma", Moore[38] indicated anaplasia and invasion, or in other words, (1) the deviation of the individual cells and acini in morphology and (2) the relation of these cells and acini to each other and to the surrounding tissues. Especially he put stress upon an irregular relation of acini to the surrounding tissues, and stated that the appearance of acini in the perineural lymphatics was a definite finding of invasive growth.

Edwards *et al.*[13] indicated three factors; they were (1) anaplasia (2) invasion and (3) structure of malignant foci as a whole. They emphasized the irregularity of the lesion as a whole in addition to the findings of individual cells and acini.

Totten *et al.*[52] considered as criteria (1) the relationship between glandular patterns and connective tissues, (2) the arrangement of the epithelium within the glands and (3) cellular details. His criteria are characterized by the cellular, especially nuclear changes.

Many others have discussed the criteria of malignancy or the histological characteristics, but these findings can be summarized to anaplasia and invasiveness. The author regarded these two as the criteria of malignancy. Cases which showed both anaplasia and invasiveness were naturally diagnosed as carcinoma and cases with definite sign of either one of two findings were also considered as carcinoma, while cases with no definite sign of two findings were excluded, even when they appeared to be malignant.

In the following these criteria will be discussed in details.

A) Anaplasia: Carcinoma of the prostate, even in a clinically manifest case, often shows a well differentiated adenocarcinoma and mitoses are rarely seen. Many reporters[7,17] stated that in latent carcinoma mitotic figures are also scarcely seen and mild anaplastic change is noted, and that these cytological changes could be little help for diagnosis. Totten *et al.*[52], however, found some differences between malignant cells and normal ones, examining surgical specimens fixed in Buin's solution. They stated that the most prominent changes were the presence of relatively large, sometimes multiple, deeply stained nucleoli, and that the nuclear chromatin was usually sparse or absent in the central portion, being concentrated at the nuclear membranes. They considered these nuclear changes as the important findings for diagnosis.

In the author's 25 cases of latent carcinoma, the above-mentioned nuclear changes were prominent in 8 cases, and slight in 6 cases. In other words, the similar nuclear changes as Totten *et al.* described were also recognizable in about a half of cases fixed by formalin. Accordingly, contrary to many reports, these nuclear changes were considered to be important for diagnosis; however, the findings that the nuclei generally were approximately equal in size and that miotsis was seldom seen were in accordance with many other reports.

Carcinoma cells are various in size and shape, showing all the transition from relatively large cylindrical or cuboidal cells with abundant clear cytoplasm to relatively smaller ones with scanty but deeply eosinophilic cytoplasm, although no changes characteristic to carcinoma were observed in cytoplasm itself. Bibus and Walthard,[54] however, observed the double reflactory fatty-granules in cytoplasm of malignant cells more abundantly than normal.

Another feature of anaplasia is a change of glandular acini. Most of the carcinoma cells preserve the ability of acinus-formation, and show a well differentiated adenocarcinoma, but each acinus is much smaller in size and more irregular in shape than normal. Moreover, acini are often converted to the form of cellular strangs or masses without any lumina, and sometimes even to the individual cells scattered in the stromal tissue. These various forms are usually mixed irregularly at various proportion in same lesion. On the contrary, small acini of a benign lesion are similar in shape and do not take any variation mentioned above.

According to Lewis[33] and Totten *et al.*,[52] a normal glandular acinus often reveals a differentiation of basal cell layer with deeply stained plane nucleus, while a malignant acinus does not. As differentiation of basal cell layer can not always be proved in benign acini, a lack of differentiation of basal cells is not a sufficient condition but only a necessary one for malignancy.

As Moore[38] observed, in the glandular acini composed of carcinoma cells, there can be seen neither architecture such as corpora amylacea and stone, nor papilla formation of the acini.

B) Invasion: Many have emphasized that invasion was a far more important sign of malignancy than cytological changes for diagnosis of prostatic carcinoma. The opinion is quite agreeable, but in an organ like prostate where glandular acini and connective tissues are normally intermingled, it is often very difficult to determine whether the acini are invasive or not.

Moore[38] and Andrews[5] thought that the relationship between acini and the surrounding tissues must be the most important sign to determine its invasiveness. Normal glandular patterns are surrounded by thin membrane of collagenous tissue called stroma propria or limiting zone, and around stroma propria are stromal connective tissues with muscle fibers, elastic fibers and blood vessels. The pattern is still preserved in benign nodular hypertrophy, and stroma propria thickens collagenously in the prostate of senile atrophy. In malignant carcinomatous foci, however, this relation is no longer seen. The glandular acini and cell masses are irregularly distributed with no relation to muscle fibers, connective tissue or elastic fibers. Moreover, these acini and cell masses may be adjacent to them, or may ever interrupt or infiltrate them. Andrews[5] said that carcinoma was suspicious when acini were surrounded by muscle fibers without intervening connective tissue or when acini showed "back to

back" arrangement, and that the "budding-off" of the cells into the muscle fiber could be adopted as a finding of invasiveness.

According to the author's observation, however, a similar picture can be sporadically seen even in non-malignant glandular acini. Therefore the author is of the opinion that it can be diagnosed to be invasive if the pictures are seen to some extent.

Baron and Angrist[7], and Edwards *et al.*[13] stated the importance to observe a lesion as a whole. Indeed, by low power magnification, very small and irregular glandular structures are arranged closely to each other without any lobular pattern in a lesion, where normal glandular acini are seen here and there. At the peripheral portion these small acini or cell masses are extending into the stromal connective tissues between normal acini, just like in a form of dendrite or processus, without any evidence of compressing the surrounding tissue.

Moore[28] observed the presence of these small acini in the perineural lymphatic spaces in 75% of his small carcinomas and laid stress on it as a conclusive evidence of invasion. In the author's 25 cases, these pictures were found in 12 cases. This is especially frequently seen in a lesion arising near the capsule in posterior portion of the prostate, even the lesion is very small. It is no doubt that the finding shows the invasive growth of these small glandular acini.

As a definite sign of invasion, vascular and capsular invasion are indicated by many workers[19][38]. Frank[19] recognized the capsular invasion in 52 cases and vascular invasion in 26 respectively among his 69 cases of latent carcinoma. In the author's 12 cases, carcinoma cells and blood vessels seemed to be related to each other intimately, and the walls of the vessels became so thin that the vascular lumina seemed to be bounded only by a single layer of endothelial cells. But as the most of these vessels are small veins, we cannot judge from this picture to be vascular invasion. In other 13 cases, destruction of blood vessel walls was not observed.

In regard to the capsular invasion, it was so difficult to decide a borderline of capsule strictly that its precise incidence could not be obtained.

Oswald[45] stated that the perineural lymphatic invasions were frequently seen, whereas the vascular invasions were relatively rare. The author agrees with this opinion.

Since basement membrane, which is often subjected to be a criterion for malignancy, has no definite rule in the normal prostate, its break-off cannot be regarded as a diagnostic criterion of prostatic carcinoma.

RESULTS

According to the criteria for malignancy mentioned above, 25 cases (10.9%) of latent carcinoma were found in 229 prostates. The results obtainted will be discussed below.

1) Extension and gross appearance.

The size of the lesions ranged from 2×2 mm in the smallest to 30×30 mm in the largest. No lesion could be diagnosed as carcinoma macroscopically. Examining the tissue embedded in paraffin after the diagnosis had been established, it seemed difficult to distinguish these lesions from the surrounding tissue.

2) Age-incidence.

Table IV shows the age-incidence of 25 cases. In the 40's, only one case was found (45 years and 3 months old). The incidence increases with age, reaching to 23% in 70's. It is well known that prostatic carcinoma has its highest incidence among the aged person, but in the author's 4 cases over 80 years old, there was no case of latent carcinoma. According to many reports, the incidence is increasing with the age. As the author's cases are relatively small in number, definite conclusion must be reserved.

TABLE IV. Age Incidence of Latent Prostatic Carcinoma

Age group	No. of cases studied	No. of cases with latent carcinoma	Incidence (%)
40-44	22	0	0
45-49	23	1	3.9
50-54	39	2	5.1
55-59	40	2	5.0
60-64	33	4	12.1
65-69	33	8	24.2
70-74	23	7	30.4
75-79	12	1	8.3
80-84	2	0	0
85-89	2	0	0
total	229	25	10.9

3) Site of origin.

The prostate is commonly devided into 5 lobes, namely, anterior, middle, posterior, and two lateral lobes. The anterior lobe is the portion anterior to the urethra, the posterior lobe is the portion posterior to the urethra and caudal from the ejaculatory duct, the middle lobe is the portion between the urethra and the ejaculatory duct, and the lateral lobe is the portion lying lateral to the urethra and connecting the anterior and posterior lobe. According to Lowsley, each lobe is embryologically different in origin and in fetal period, often even in the adult, divided by septa, and each lobe has its own excretory duct. But others say that the excretory duct is independent of either lobe. In the adult

prostate, however, it is impossible, not only macroscopically but also histologically, to recognize the lobar differentiation, especially in the posterior and the lateral portion having the same histological structure. Therefore, the author divided the prostate, in accordance with Kahler[22], into 5 portions as shown in Fig. 1. On the other hand, Adrion[2] divided the prostate into inner and outer glands. The inner glands are glandular tissue lying inside the layer of longitudinal or oblique muscle fibers near the urethra, the excretory ducts of which open into the portion distant from the center of seminal colliculus, and the outer glands are original prostatic glandular tissue outside the layer of muscle fibers, the excretory ducts of which open to the part near the center of colliculus.

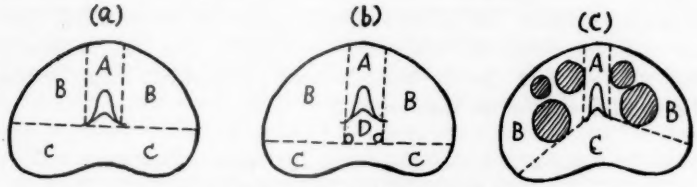

Fig. 1. Schema of dividing into lobes.
(a) and (b): In a case without nodular hypertrophy.
(c): In a case with nodular hypertrophy.
A: anterior lobe
B: lateral lobe
C: posterior lobe
D: middle lobe

In two of the author's cases, two malignant foci were found separately in one prostate, so malignant foci were 27 in total.

In the other two cases, the foci were so vastly invasive that it was impossible to decide the definite site of origin. In the remaining 25 foci, 12 were found in the posterior lobe, 11 in the lateral lobe, and 2 in the anterior lobe. No primary foci were found in the middle lobe. Fig. 2 shows these distribution in schema. All the primary foci were found in the outer glands, especially in peripheral portion near the capsule and no primary foci originated in inner glands.

Gaynor[21] classified the site of origin of malignant foci according to their height. If the prostate is divided into upper, middle and lower parts, primary foci originated in the upper part were only 3, and the majority of primary foci was located in the middle and lower portion. This predilection shows a striking contrast to that of the benign nodular hypertrophy which usually is found in the upper portion. This may be concerned with the anatomical fact that as the excretory ducts of the lower portion of the prostate run upward to open near the center of colliculus, the prostatic secretion tends to accumulate in the lower

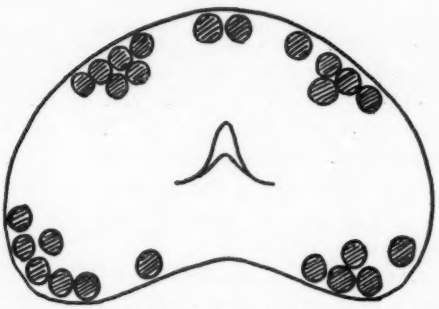

Fig. 2. Site of origin of latent prostatic carcinoma in schema.

portion due to the senile atrophy of elastic fibers and muscle fibers in stroma.

4) Relationship with nodular hypertrophy.

Albarran & Hallé[3] assumed that nodular hypertrophy would make malignant change, but at present, the generally accepted opinion is that these two conditions are independent of each other and nodular hypertrophy does not transform itself to carcinoma.

In author's cases, as shown in Table V, incidence of carcinoma has no relation to presence or absence of nodular hypertrophy. Nodular hypertrophy is rather a physiological change over the age of 50, and carcinoma also occurs in the aged person. Therefore, it is no wonder that carcinoma is often found in the prostate with nodular hypertrophy. Furthermore, in the prostate in which both nodular hypertrophy and carcinoma were found, they were different in location. Even in the 2 cases in which carcinoma extended to inside the nodule, the main focus of carcinoma was outside the nodule. Thus, any finding which appears to show the transition from nodular hypertrophy to carcinoma was not seen in author's cases.

Table V. Relationship of Prostatic Carcinoma to Benign Prostatic Hypertrophy

Age group	Prostates with hypertrophy			Prostates without hypertrophy		
	Cases studied	Cases with carcinoma	Incidence (%)	Cases studied	Cases with carcinoma	Incidence (%)
40-49	10	0	0	35	1	2.9
50-59	29	2	6.9	50	2	4.0
60-69	40	9	22.5	26	3	11.5
70-79	29	6	20.7	6	2	33.3
80-89	4	0	0	0	0	0
total	112	17	15.1	117	8	6.8

5) Morphology.

Latent carcinoma of the prostate shows various histological pictures. Totten *et al.*[52] classified them into 1) cribriform arrangement, 2) diffuse individual cell infiltration, 3) medium-sized-gland carcinoma and 4) small gland carcinoma. Frank[19] divided carcinoma cells into clear cell, dark cell, anaplastic cell and squamous cell, and classified histological pictures into 1) small acinar, 2) solid trabecular ,3) cribriform, 4) intracystic and 5) scirrhous type. Edwards *et al.*[13] classified them into 1) duct type, 2) tubulo-alveolar type, 3) intratubular type, and 4) undifferentiated or anaplastic type.

Even in the same specimen, the histological pictures are not always uniform, but various histological pictures are intermingled. All of the author's cases were mainly small acinar adenocarcinoma, but the incomplete cribriform arrangement was mixed in 2 cases, and undifferentiated type with little tendency to acinar formation was found in 1 case. These adenocarcinomas can be classified into the duct type composed of dark cell and the tubulo-alveolar type composed of clear cell. Both types are mixed at various proportion. It is reported that squamous cell carcinoma can occasionally be found, but there was none in the author's cases.

6) Secretion of carcinoma cell.

The glandular acini of carcinoma are often empty, but homogenous secretion stained in eosin is often recognized. This secretion, like the contents of the benign acini, shows positive PAS-reaction. This PAS-positive substance is often found as fine granules in cytoplasm of carcinoma cells too. This shows that carcinoma cells have nearly physiological secretion. The contents of carcinomatous acini and those of physiological ones show no difference in H-E staining and PAS-reaction, but a little difference in mucicarmine-staining. Frank[19] seems to be the only reporter that performed systematically mucicarmine-staining of the prostate. According to his report, all the normal glandular acini were negative in mucicarmine-staining, and positive result was obtained only in malignant acini and "postsclerotic hyperplasia". This fact seems to be one of the basis of his opinion that hyperplasia can transform itself into carcinoma.

The author, too, stained mucicarmine in 25 cases which seemed to be physiologic and whose ages were nearly as same as the carcinoma cases. In 15 cases, the distended glandular acini which contained mucicarmine-positive contents were sporadically observed and the epithelia of the excretory duct which had strongly positive granules in their cytoplasm were seen near the urethra although the majority of contents of glandular acini were mucicarmine-negative. In the malignant foci, however, mucicarmine-positive substance was often found in the glandular lumina or in cytoplasm of carcinoma cells. The most common type was the appearance of mucicarmine-positive substance in the form of

membrane or streak in the glandular lumina, especially near the wall of carcinoma cells. In 15 of 25 cases with latent carcinoma, such findings showing a striking difference from normal glandular acini were recognized. Especially in 3 cases, these findings were marked. As mucicarmine-positive substance was recognized in the so-called Frank's "postsclerotic hyperplasia", and accidentally in the normal prostate too, it cannot be a characteristic change for carcinoma cells, but it seems to be indicative of secretion of carcinoma cells being a little different from the normal. It is interesting that, latent carcinoma of the prostate often reacts mucicarmine-positive, in spite of the fact that mucoid carcinoma is rare in clinically diagnosed prostatic carcinoma. Among 5 cases died of prostatic carcinoma, the morphological pictures of which were adenocarcinoma or carcinoma simplex, there were two cases which were mucicarmine-positive. Therefore, it is supposed that even in cases which do not reveal mucoid carcinoma, the mucicarmine-positive substance may often be recognized.

7) Multicentric origin.

Latent carcinoma of the prostate is often reported to originate multicentrically.

Moore[38] recognized 3 malignant foci in a prostate in 4 cases, 2 in 7 cases of his 52 cases, and Gaynor[21] recognized 2 to 4 malignant foci in 34 cases of his 162 cases, and found that their morphological pictures were different. Gaynor attributed this fact to multicentric origin. Of the author's cases, 2 malignant foci were found far from each other in a prostate in 2 cases. In 1 case, malignant foci were found in the posterior and the lateral lobes, but their morphological pictures were so similar that it was questionable whether they were multicentric or not. In another case, 2 malignant foci were found in the anterior and the lateral lobes, and their morphological pictures were a little different from each other, so they were supposed to have the multicentric origin.

8) Relationship between latent carcinoma of the prostate and carcinomas in other organs.

Latent carcinoma of the prostate is often associated with malignant tumors in other organs. The statistical observation of the 229 cases examined is shown in Table VI. Malignant tumors were found in other organs in 13 of the 25 cases of latent prostatic carcinoma. Judging from the fact that 131 of the total 229 cases died of malignant tumors, latent carcinoma of the prostate and malignant tumors in other organs are co-existent only by chance. The reason why latent carcinomas of the prostate are often found in the case of hypertensive vascular changes of cerebrum, is based on the fact that such cases are of the aged persons.

Table VII shows the site of malignant lesion in other organs which caused

TABLE VI. Relationship of Latent Prostatic Carcinoma to Fatal Disease

Fatal disease	No. of cases examined	No. of cases with prostatic carcinoma	Incidence (%)
Malignant tumor	131	13	9.9
Cerebral change due to vascular damage	15	6	40.0
Inflamatory disease	34	2	5.9
Others	49	4	8.2

death of patients and incidence of latent carcinoma. As shown, there is no significant relationship between them. Moore[38] stated that struma showed a high rate of association with latent prostatic carcinoma, and Gaynor[21] found a high percentage in carcinoma of the colon. In the author's cases, lung cancer had slightly higher percentage, but this may be not any significant meaning because of small number of cases examined. Baker[6] stated the relationship between the prostatic carcinoma and leukemia, but in the author's cases only one case of multiple myeloma was associated with latent carcinoma of the prostate, but none in leukemia.

TABLE VII. Relationship of Latent Prostatic Carcinoma to Malignant Tumors in Other Organs

Malignant tumor in other organs	No. of cases examined	No. of cases with latent prostatic cancer	Incidence (%)
Carcinoma of stomach	34	2	5.9
〃 lung	30	6	20.0
〃 oesophagus	13	2	15.4
Hepatoma	7	1	14.3
Cholangioma	7	1	14.3
Carcinoma of pancreas	5	0	0
Sarcoma	5	0	0
Leukemia	6	0	0
Multiple myeloma	6	1	16.6
Others	18	0	0

DISCUSSION

At present, prostatic carcinoma is classified into the following three types from the clinico-pathological point of view.

(1) Clinically manifest carcinoma.

This has local symptoms of prostatic carcinoma, and is relatively easy to diagnose. Normal structure of the prostate is destroyed. Most of this type show well differentiated adenocarcinoma.

(2) Occult carcinoma.

In spite of extensive lymphatic and bone metastasis, occult carcinoma has few local symptoms and malignant focus in the prostate is small. Most of this type show pictures of adenocarcinoma or carcinoma simplex with predominent anaplasia.

(3) Latent carcinoma.

This is found by chance microscopically at autopsy or at the operation of hypertrophy in the prostate which has no symptoms or findings of prostatic carcinoma. Usually foci are small and most of them show a picture of well differentiated adenocarcinoma.

Although the classification mentioned above is quite a convenient and not a strict one based on a definite criteria, many investigators have reported unexpectedly high incidence of latent carcinoma.

On the other hand, some are in opposition to the existence of latent carcinoma. Gloggengiesser[22,23] recognized one case of atypical proliferation in 200 prostates of autopsy cases, but it could not be defined latent carcinoma, and 24 cases which appeared to be invasions of carcinoma, were senile atrophy or compression atrophy. He concluded that there were no carcinomas without clinical symptoms and that most of the cases ever reported as latent carcinoma in literature might not be carcinoma morphologically. Büchner also stated that he could not find even a case of latent carcinoma in 50 autopsy cases.

But in the author's examination, there were some cases which showed a histological finding compatible with carcinoma, but without any clinical sign in life time. These foci were located within the prostate. Therefore the author affirms the existence of latent carcinoma and thinks it exists more frequently than expected.

As stated above, these malignant foci have many names. And even if reported as latent carcinoma, it does not always have the same meaning. Totten *et al.*[52] took it for metastasizing carcinoma unable to diagnose clinically, on the other hand Lewis[33] and Allemann[4] understood it as a precancer. The author, according to Andrews,[5] who first used the name of latent carcinoma, thinks it proper to use the name as a carcinoma which does not show any clinical symptom of prostatic cancer notwithstanding its histologically malignant findings, mostly located inside the prostate.

In the following the results will be discussed comparing with literature. (1) The incidence of latent carcinoma varies according to each reporter. Main factors influencing the incidence may be as follows. (a) Materials : The incidence is generally low in the surgical specimens of benign hypertrophy. This is only natural because surgical capsule where latent carcinoma originates, is usually left in the operation of hypertrophy. Bibus, Allemann,[4] or Ichikawa[29] and Ito[31] in our country reported the incidence to be 5% or so by surgical specimens.

Obviously the incidence becomes higher in materials containing more aged persons, and if the author's cases are limited over 50 years old, the incidence becomes 13%.

(b) Methods: The foci of latent carcinoma are so small that they are liable to be overlooked in only one preparation. Rich[47] stated that he had found carcinoma in 14% of preparations made as a routine work at autopsy, and that elaborate examination might reveal more.

Baron and Angrist[7] made an examination of 2 series of preparations, and reported that the incidence is 14.8% in the examination of one preparation made by random section and 46% by step section. There is a great difference between them. Walthard[54] reported that the incidence was 30% in an examination of serial sections. The author selected as complete materials as possible and examined by step section. But in the aged persons materials were so few that the imcomplete materials were also included, so if the examination was based on the materials collected and conserved for this purpose, the incidence would have been higher.

(c) Criteria of malignancy: It is obvious that the incidence is dependent greatly upon the criteria of malignancy, which vary according to each reporter. Edwards *et al.*[13] reported the 15 cases, diagnosis of which was different between different observers they consulted.

It is true that there are some benign changes of small glandular patterns, and at the same time it is also true that there exists a carcinoma with small glandular patterns, obviously invasive. However, there are all transitional forms between the two. Therefore, the diagnosis of each case cannot avoid the examiner's subjectiveness. It is thus impossible to make a linear classification histologically between malignant and benign, and accordingly all the doubtfull cases were excluded from carcinoma, and dealt as benign for statistical purpose in this paper.

As stated above, the incidence varies according to several factors, so we cannot understand every report in a uniform manner. But the author thinks it is certain that there may exist latent carcinoma far more frequently than expected from the death rate of prostatic carcinoma.

(2) As for the site of origin of latent carcinoma, the posterior lobe is mentioned in general as most frequently prevalent.

Moore[38] stated that the incidence in the posterior lobe was 73.5%, the lateral lobes 8.8% and the anterior lobe 14.8%. Gaynor[21] reported that the incidence in the posterior lobe was 60.6%, the lateral lobes 9.8% and the anterior lobe 29.1%. Baron and Angrist[7] stated that 4 cases were found in the posterior lobe, 9 cases in the lateral lobes, and the other 10 cases were difficult for them to determine which lobe was their origin.

But differentiation of lobes is difficult both macroscopically and microscopically and Kahler[32], who differentiated lobes in the same way as the author, reported the fact that he had found a little more cases in the lateral lobes than in

the posterior lobe. In the author's cases, the incidence was almost the same in the posterior and the lateral lobes. All reporters agree that latent carcinoma is most prevalent in the peripheral portion near the capsule, and it has a tendency to origin postero-laterally and antero-laterally rather than median portion. The results of this report are in complete accordance with this opinion. This portion seems to be situated at the embryonal septi by Lowsley, and changes of senile atrophy seem to occur at the earliest stage, and at the same time a picture of proliferation of small glandular patterns similar to carcinoma is often found there.

Moore[38] drew inference that carcinoma might originate from the once atrophied glandular epithelia from the fact that such atrophic changes were conspicuous in the cases in which carcinoma was found.

But Andrews[5] presumed that proliferative changes had some relationship with carcinogenesis, because in such cases atrophy could not be said to be especially conspicuous, but on the contrary, around the malignant foci hypertrophic glandular acini were often found to be prevalent. Edwards *et al.*[13] also recognized such hyperplastic acini around malignant foci in a few cases. From the author's cases, too, atrophy could not be said to be conspicuous in cases that carcinoma originated, and no atrophic change could be found in the bengin glandular acini scattering in the malignant lesion. In view of the fact that glandular hyperplasia such as described by Andrews is found in 3 cases, the proliferative changes or the stimuli what had caused the proliferation might be partially responsible for the carcinogenesis.

Foot *et al.*[15] and Edwards *et al.*[13] divided the glandular acinus into acinar, tubular and ductile parts, and presumed that the duct type carcinoma originated from ductile part and the tubulo-alveolar type from the epithelia of acinar or tubular part. But the origin, even in a very small focus, is unknown from which part of the glandular patterns it originated and that these two types are found mixing in a same focus, so the difference of types does not seem to be dependent only to the part of the glandular patterns.

Other factors such as inflammatory changes, concretion, and arteriosclerosis were not recognized to be related to carcinogenesis.

(3) The most important and interesting question will be the relationship and the difference between latent carcinoma and clinically manifest tumors.

Many opinions have been stated on this problem, but none of them has a positive proof.

Bibus said that there was too wide a discrepancy between the death rate and the incidence of latent carcinoma to regard latent carcinoma as quite the same with clinically manifest carcinoma, and v. Meyenburg[35] concluded that the carcinoma as histological changes should be differentiated from the one as a disease. But Frank[14] stated, basing upon the results of animal experiments by Berenblum *et al.*[9] that, of the developmental "stages" of carcinoma, the

"praeclinical stage" might coincide with latent carcinoma, and for the reasons to be latent, he said that the characteristics of carcinoma cell itself, conditions on the part of the host, or both might be concerned and that hormone might partially be related.

The growth-rate of latent carcinoma, by Kahler's opinion, is very slow, but it will grow to become a clinically manifest carcinoma in a fully long period. But, as most of them originate in the aged persons, they are found to be latent carcinoma. Therefore, Kahler[32] stated that latent carcinoma would become a clinically important problem as the prolongation of life expectancy.

Latent carcinoma generally shows a well-differentiated histological pictures which seem to be comparatively benign, and the growth-rate seems to be slow, so it can exist without any clinical symptom for a long time. Considering the fact that in Stearns and Gordon's report[50] of their experience in 3 cases, the histological picture is said to have changed into a less differentiated picture after hormonal therapy, some changes of conditions may accelerate the growth-rate and cause latent carcinoma to develop into clinically manifest carcinoma. But whether latent carcinoma remains latent indefinitely, or whether it will grow to become clinically manifest, or whether every clinically manifest carcinoma passes through this stage are problems unable to conclude only by means of the histological examination. To study these problems, it is quite necessary to pursue many cases for a long period.

The rapid increase of the death-rate of prostatic carcinoma in our country is so conspicuous compared with carcinomas in other organs, that it has attracted a public attention. This phenomenon can easily be explained if we assume the possibility that the carcinoma hitherto recognized as latent may develop into a clinically manifest one with the striking prolongation of life expectancy in our country. But at present, when we know nothing about the true character and the causes of latent carcinoma, the reason why the incidence of latent carcinoma is almost the same as in other countries in spite of the lower death-rate of prostatic carcinoma must be said to be unknown.

CONCLUSION

The author examined, by step section, the prostates of 229 male autopsy cases over the age of 40, who did not have any symptom of prostatic carcinoma in their life time, and did not be aware of prostatic carcinoma macroscopically.

The results of the examination are:

(1) The author could find malignant foci in 25 cases (10.9%) among the total 229 cases. Each focus was located inside the capsule and could be designated as latent carcinoma. The incidence of latent carcinoma in our country is almost the same as in Europe and America in spite of the lower death-rate of prostatic carcinoma.

(2) The site of origin of latent carcinoma was the posterior lobe in 12 cases, lateral lobe in 11 cases and anterior lobe in 2 cases. The incidence in the posterior lobe and lateral lobes was almost the same. Each of them originated near the capsule and postero-laterally or antero-laterally from the median portion and only a few cases were found upward the seminal colliculus. And in 2 cases 2 foci were found distant from each other.

(3) Most of the histological pictures were small acinar adenocarcinoma, and in 2 cases incomplete cribriform arrangement and in 1 case an anaplastic type of little tendency of acinar formation were found. There was no squamous cell carcinoma. In the nuclei of carcinoma cells there appeared nucleoli and paleness of the center of the nuclei, the irregular relationship between malignant acini and their surrounding tissues was considerably characteristic, and the invasion into the perineural lymphatics often recognized was a definite index of invasiveness, but the vascular and capsular invasion were rare.

(4) Carcinoma was often associated with nodular hypertrophy, but there was no causal relationship between them, and moreover no direct relationship with atrophy. However, hypertrophic glandular acini around the malignant lesion were at times recognizable and proliferative changes seemed to be more related rather than atrophy.

(5) In malignant foci, the mucicarmine-positive substance which cannot be recognized in the normal tissue is often recognizable. Especially in 3 cases it was very conspicuous.

(6) Any conclusion could not be drawn as for the relationship between latent carcinoma and clinically manifest carcinoma, the causes to be latent, and the reasons of lower death-rate of prostatic carcinoma in our country.

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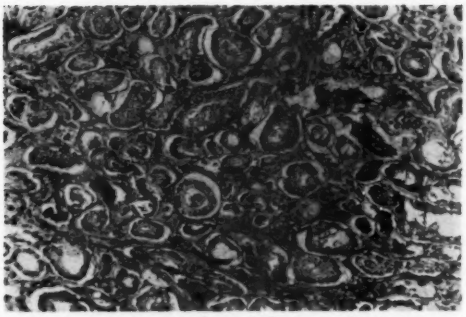

Fig. 3. Most common histological feature of latent prostatic carcinoma. Note the irregular arrangement of small acini to each other, and the irregularity in their shape.

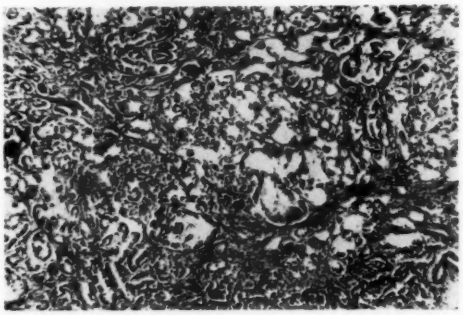

Fig. 4. Destruction of stromal connective tissue in the latent carcinomatous focus.

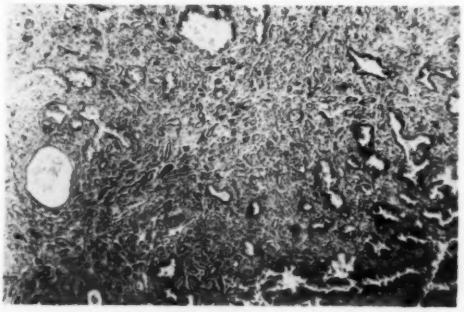

Fig. 5. Carcinomatous infiltration into the stromal connective tissue between benign acini at the peripheral portion of carcinomatous lesion.

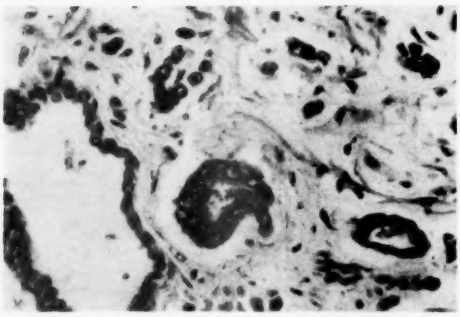

Fig. 6. Nuclear changes of the latent carcinoma cells. The appearance of nucleoli and the paleness of central portion of nuclei are noted.

Fig. 7. Common type of perineural lymphatic invasion of cancer cells.

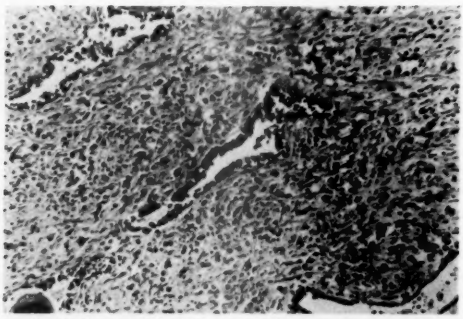

Fig. 8. Individual-cell-infiltration of anaplastic cells with little tendency of acinus formation.

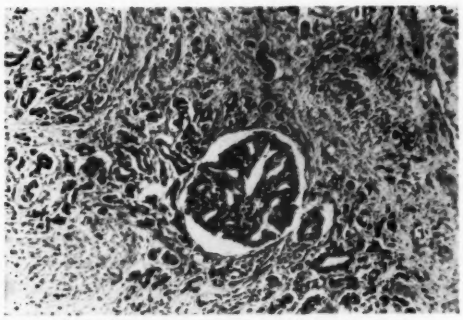

Fig. 9. Cribriform arrangement of latent carcinoma cells.

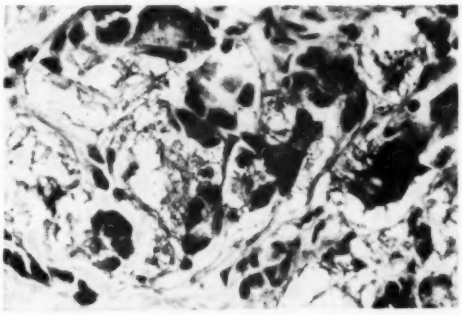

Fig. 10. Mucicarmine Staining. Mucicarmine positive substance can be seen in a form of membrane or streak in the glandular lumina of latent carcinomatous focus.

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Studies on Radiation Disturbances during Treatment of Uterine Cancer. Especially on the Radiopathogenic Substance and New Therapy

By

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We often experimence the radiation disturbances (RD hereunder), that is leucopenia, thrombocytopenia and sympathetic nervous syndrome, while administering radiotherapy following operation of uterine cancer and such malignant neoplasms and against advanced malignant female genital tumors. They sometimes become so pronounced as to necessitate suspension of the therapy at times even forcing us to waste the most precious time for inhibiting growth of the neoplasm. The present authors have made some researches upon the pathogenic factor that induces such RD, and a summary of the results obtained hitherto is presented in the following.

I. EXPERIMENTS ON RD

1. On the Radiopathogenic Substance

1) Investigation on the existence of radiopathogenic substance

After rabbits were irradiated serially with X-ray to induce RD, blood was sampled from them and analysed into 3 parts of blood cells, serum and plasma. Upon injecting these blood parts separately in normal rabbits, it was found that the blood cells had the strongest effect in inducing RD. The substance supposed to give this effect of causing RD to blood cells will be called radiotoxin hereunder.

2) Extraction of radiotoxin

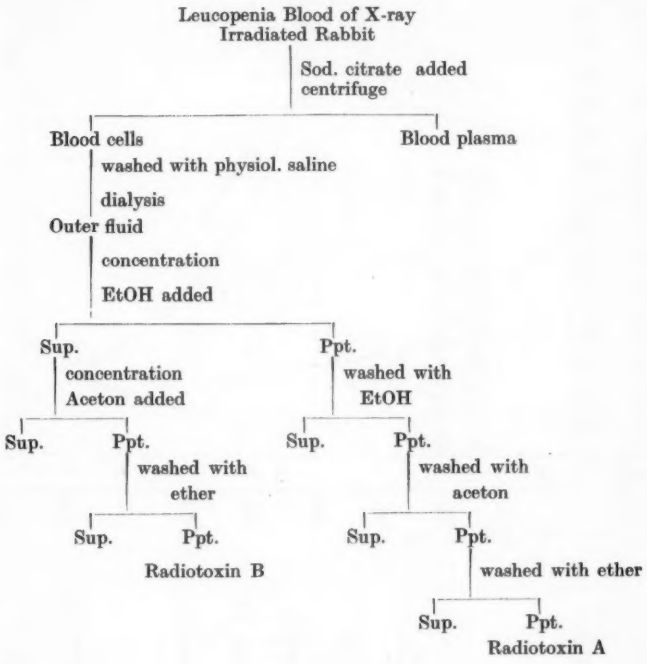
Blood cells sampled from the rabbits that showed the severest symptoms of RD were subjected to dialysis, fractional extraction with alcohol, ether and so forth, as shown in Table I, and radiotoxin was successfully isolated. This is a low molecular substance but the chemical constitution and characteristics are now under investigation.

3) Action mechanism of radiotoxin

Intravenous injection of radiotoxin induces pronounced decrease of leuco-

九嶋勝司, 吉崎　宏

TABLE I



cytes, as shown in Fig. 1, but when it was injected with pregicil, an autonomic nerve blocker, the decrease of leucocytes was inhibited and when administered in mixture with pereston N, a conjugating detoxicant, the decrease of leucocytes was not inhibited, as shown in Fig. 2, but only the reincrease to normal level was accelerated. The decrease of leucocytes by radiotoxin, therefore, seems to be induced through the intermediary action of the hypothalamus, the seat of centers for adjustment of blood cells and of the autonomic nerves.

4) Confirmation of the radiotoxic effect

Blood cells sampled from rabbits receiving radiotoxin in serial injections, when separated from plasma and injected in normal rabbits, cause propounced decrease of leucocytes. This result convinced us of the existence of some component in such blood cells responsible for the RD.

5) Histological changes in entrails following injection of radiotoxin

Following injection of radiotoxin, histological changes were chiefly observed in the detoxicating and excreting organs, the liver and the kidneys, only slight changes being found in the other organs.

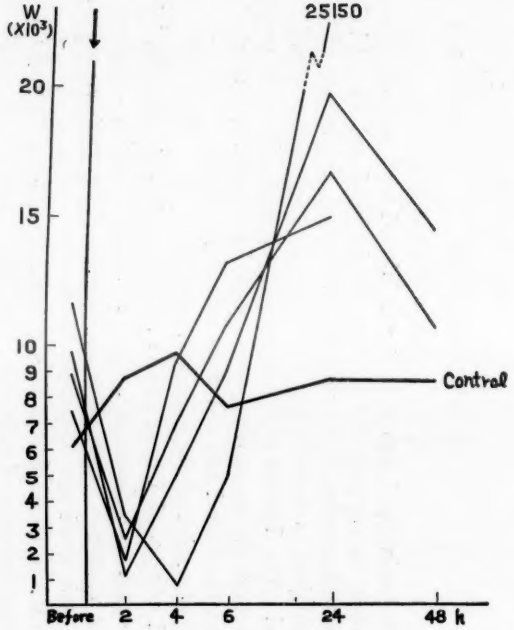


Fig. 1. Experimental results of radiotoxin a injection (20~50 mg.). Total leucocyte count.

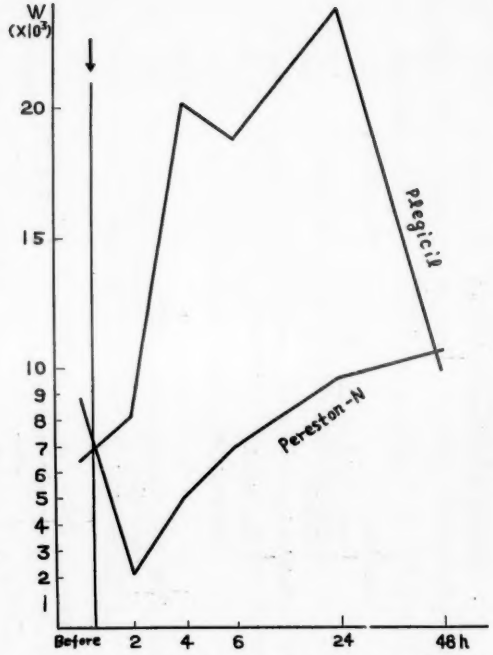


Fig. 2. Results of mixed injection of radiotoxin and Plegicil or Pereston N. Total leucocyte count.

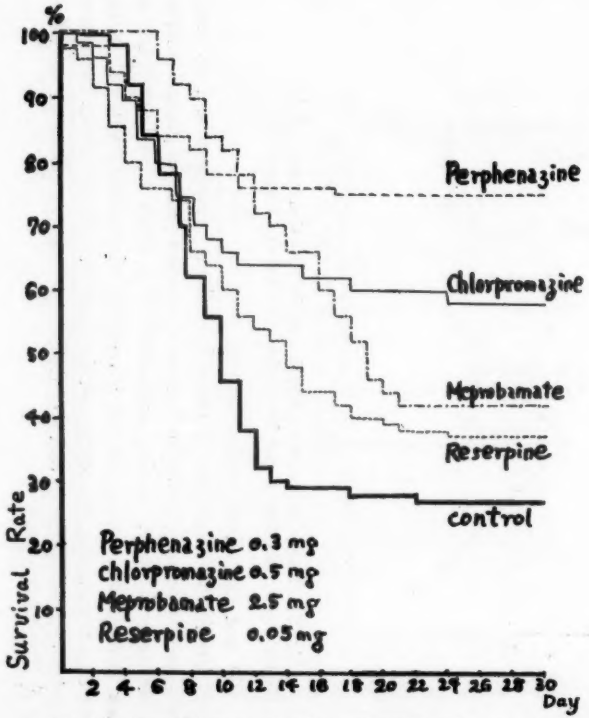


Fig. 3. Survival rate of mice pretreated with tranquilizers. 50 mice per group. Irradiation : 500 γ.

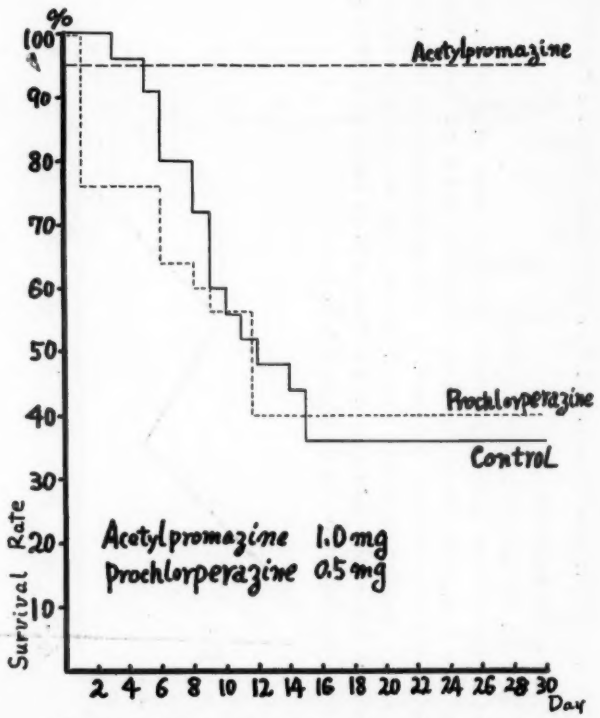


Fig. 4. Survival rate of mice pretreated with tranquilizers. 50 mice per group. Irradiation : 500 γ.

2. Causes of Radiation Sickness and Decrease of Leucocytes and Platelets

A. Animal Experiments

1) RD and the autonomic nerve system

Various tranquilizers (automonous nerve blockers) were administered in one dosis each to mice one hour before X-ray irradiation, and it was found that the survival rate was obviously enhanced by such pretreatment, as shown in Figs. 3 & 4. Another experiment showed that the ChE activity in the hypothalamus, the seat of the autonomic nerve center, and its marginal systems is altered by X-ray irradiation, as shown in Fig. 5.

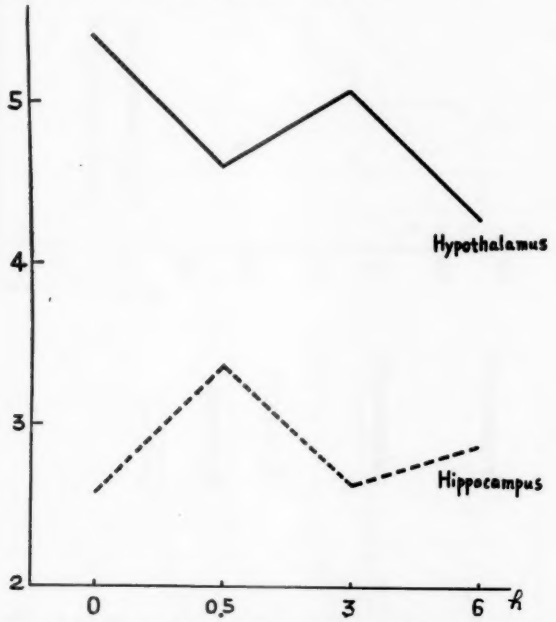


Fig. 5. Change in ChE concentration in the hypothalamus and the hippocampus following X-ray irradiation. Ordinate : ChE activity.

2) RD and the adrenocortical system

X-ray irradiation constitutes a stress for a living organisms, and, as many other non-specific stresses, probably induces a reaction chain of general adaptation syndrome in a body, causing activation of a series of endocrine defense processes through the agency of the hypophysis-adrenocortex system ; acting upon such an assumption, we pretreated the experimental animals as above with adrenocortex-activating agents and found the survival rate enhanced, as shown in Fig. 6.

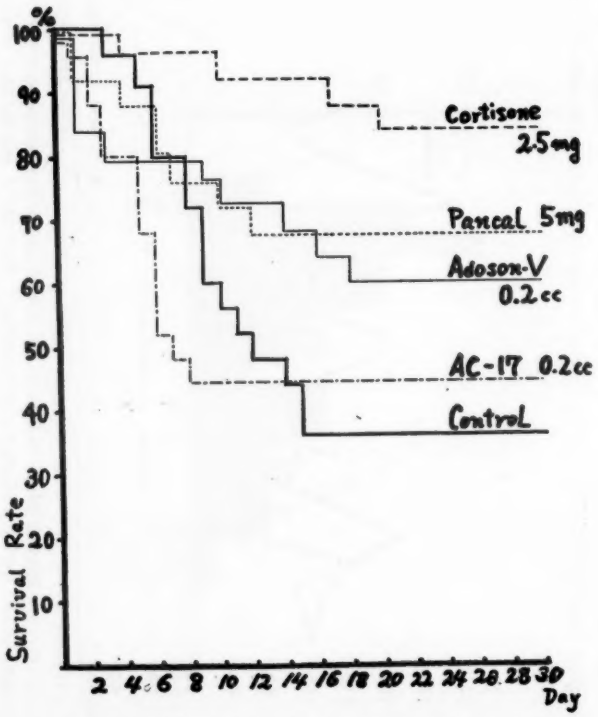


Fig. 6. Survival rate of mice treated with some drugs before or after irradiation. 25 mice per group. Irradiation : 500 γ.

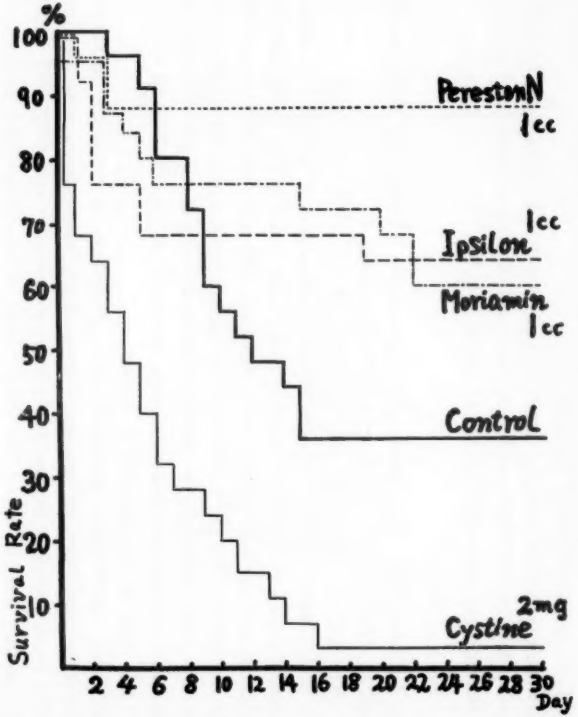


Fig. 7. Survival rate of mice treated with some drugs before or after irradiation. 25 mice per group. Irradiation : 500 γ.

3) RD and detoxicating function

Experiments of administering drugs with conjugation-detoxicant, hepatotonic or hematopoietic agents were conducted with radiotoxinated mice, and again a rise of survival rate was affirmed, as shown in Fig. 7.

B. Clinical Tests

The following tests were performed to determine whether the above results of animal experiments are available for clinical purpose.

1) RD and the autonomic nerve system

The flicker values in the subjects showing symptoms of radiation troubles, as estimated with a Motokawa's[1] electric flicker-meter and reflecting the activity of autonomic nerves, were found more strongly affected than in the non-RD controls, as shown in Fig. 8; in the subjects administered up to about 30 rounds of 300 r each, the change in the flicker value was found to run parallel with the grade of radiation sickness and leucopenia, as shown in Figs. 9, 10, and 11.

2) RD and the adrenocortical system

The 17KS value[2] rises transiently with irradiation, to reach the maximum after the 5th irradiation or so, then comes down approximately to the pre-irradiation level, begins to rise again in a wavy course after about the 25th round, to reach another peak at about the 30th round of irradiation, and dropped off rapidly thereafter. The 17OHCS[3] value changed in a similar way but somewhat lagging behind the 17KS value. The change in flicker value also ran a

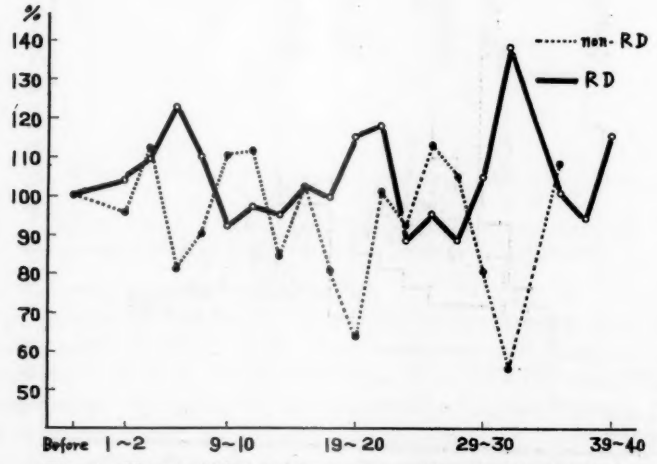


Fig. 8. Electric flicker values in cases with and without radiation disturbances during radiotherapy. Abscissa: Rounds of irradiation.

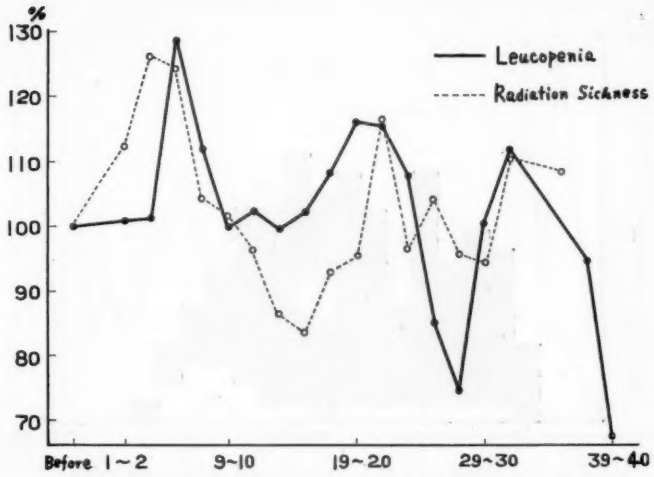


Fig. 9. Electric flicker values in cases with radiation sickness and leucopenia.

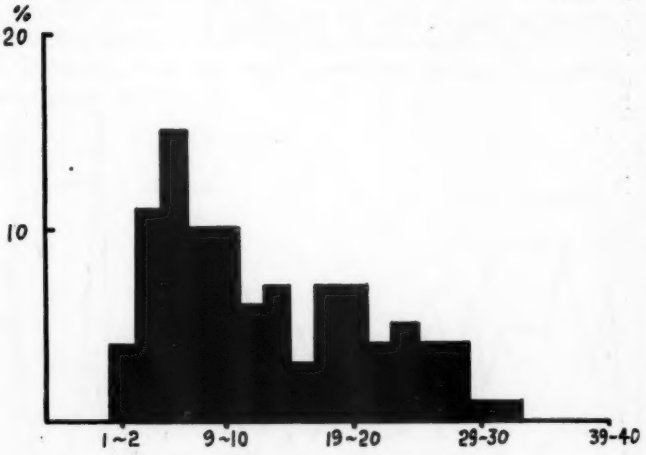


Fig. 10. Occurrence of radiation sickness.

parallel course with that of 17KS to some extent. These finding suggest that exposure to radiation, besides stimulating the autonomic nerve system, acts as a stress upon the adrenal grands, to stimulate their secretion of 17KS and 17 OHCS. Both the 17KS and the 17 OHCS values were higher in the radiation

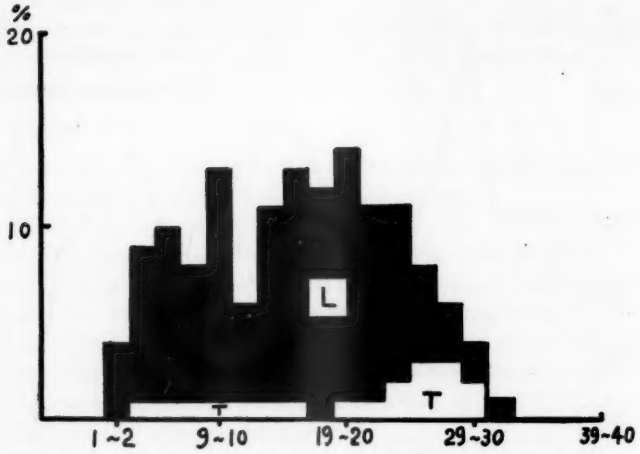


Fig. 11. Occurrence of leucopenia and thrombocytopenia.

disturbances than in the non-radiation disturbances controls (Figs. 12 and 13), and the flucutuation in 17KS value ran nearly parallel to the grade of radiation sickness, but was not related with the leucopenia.

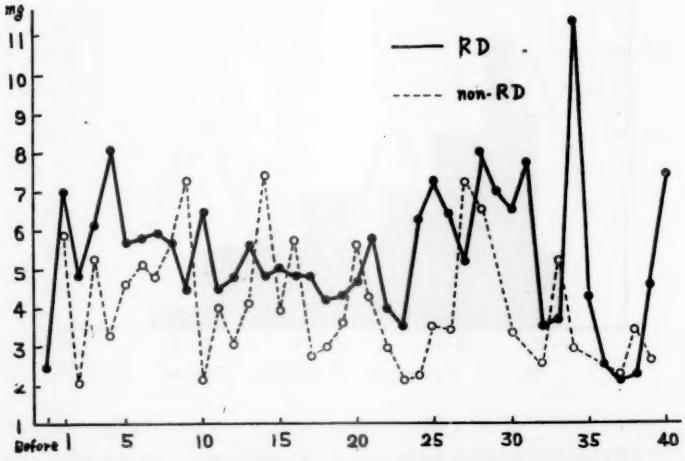


Fig. 12. Relationship between radiation disturbances and 17KS content in urine.

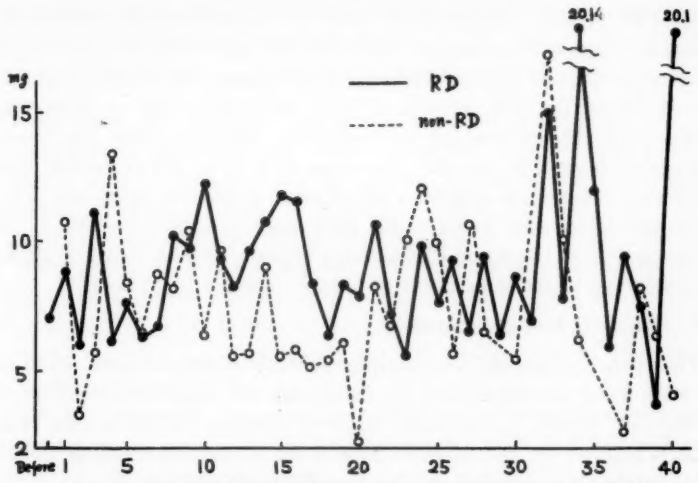


Fig. 13. Relationship between radiation disturbances and 17 OHCS content in urine.

3) RD and the enzyme systems

a. Catalase system: The catalase activity of blood cells and serum as estimated with a Stern and Battelli's[4] apparatus, apparently fell off slightly with repeated irradiation, but the trend showed no significant correlation with the presence or grade of RD.

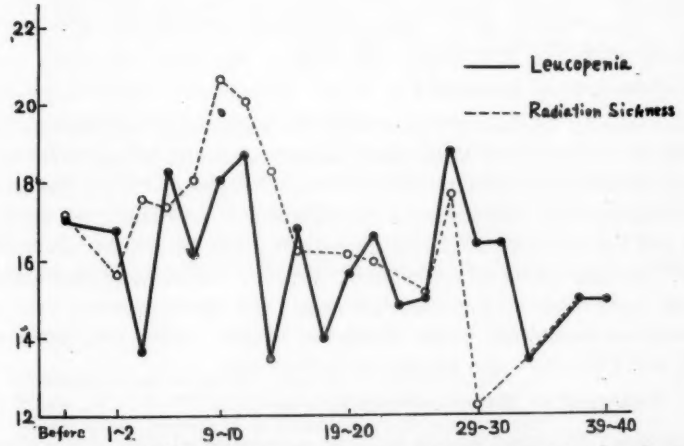


Fig. 14. ChE concentration in cases with radiation sickness and leucopenia. Ordinate: ChE activity

b. ChE system: There is as yet no incontrovertible theory on the correlation between the autonomic nerve function and ChE. On the other hand, there is an undeniable close correlation between serum ChE and the hepatic function. We found the ChE activity of blood cells and serum, as determined by Kasuga's method[5], was lowered by irradiation, especially when the irradiation was repeated beyond 30 rounds; the serum ChE value fell in parallel with the fall in flicker value in the subjects with radiation sickness or leucopenia (Fig. 14.), but the parallelism went as far as the 30th round of irradiation and no farther. The strong drop of the serum ChE activity beyond the 30th round of irradiation is probably due to the impairment of hepatic function by radiotoxin.

C. Cause of Radiation Anemia

The porphobilinogen, coproporphyrin and protoporphyrin contents in urine were found increased when the irradiation was repeated beyond 30 rounds and especially largely in the cases prone to anemia. It seems that radiation anemia is induced by the effect of large doses of irradiation acting inhibitorily on the porphyrin metabolism in the hematopoietic organs.

II. TREATMENT OF RD

1. Treatment of Radiation Sickness

Considering the frequency of radiation sickness attributable to anomaly of the autonomic nerve system, the cases were first treated with tranquilizers (perphenazine, prochlorperazine, acetylpromozine, meclizine hydrochloride, etc.) one hour before irradiation, and satisfactory results could be attained. PVP preparations, essential amino-acid preparations, pantothenic acid, ε-amino caproic acid and thioctic acid were also used.

2. Treatment of Leucopenia

The transient decrease of leucocytes at the beginning of radiotherapy seems to be due to an anomaly in the blood-cell adjustment center by radiotoxin but the leucopenia following repeated irradiation seems to be attributable to a derangement in the hematopoietic organs caused by radiotoxin. Accordingly, we make it a rule to test the hematopoietic function by cobalt-greenpole method[6] and adenine method[7], to assure chocie of best-adapted medicine. As adrenocortical hormones cortisone, predonisolone, methylpredonisolone, and dexamethasone were used. The nonhormonous drugs (cobalt-greenpole, adenine, orotic acid, aminoacids, cystine and PVL) were also employed for treatment.

3. Treatment of Thrombocytopenia

Sometimes thrombocytopenic purpura is experienced during radiotherapy, so that a decrease of blood platelets should not be made light of. For fighting

this anomaly, adrenocortical hormones are prominently efficatious; no sure efficacy can be expected from any other drug.

4. Treatment with X-rayed Blood

At this clinic, the following treatment is given to cases with leucopenia or thrombocytopenia; 100 cc of fresh blood is sampled with a syringe containing 10 cc of 10% citric acid solution, exposed to X-ray irradiation under the conditions of; generator, Toshiba KXC-18; tube voltage, 180kv; tube amperage, 20 mA; filter, Cu 0.7+Al 0.5; distance, 40 cm; irradiation rate, 66.7 r/min.; irradiation dosis, 300r, and is then injected in the same manner as in blood transfusion. This treatment has proved notably effective in 40.9% and 40.5% and less notably effective in 36.4% and 48.7% of leucopenia and thrombocytopenia cases, respectively. In some cases, the treatment was successful even when adrenocortical hormone treatment failed.

For further details on the theory and practice of the above treatments against RD, the readers are referred to our original report[7] in Journal of the Japanese Obsterical and Gynecological Society.

III. CONCLUSION

The results of our experiments summarized in the above seem to lead to the following conclusions:

Radiation disturbances are probably caused by production in the irradiated body of a substance that may be called radiotoxin, which induces first radiation sickness and leucopenia through the agency of autonomic nervous center, while the stress due to the irradiation deranges the function of endocrine organs through the agency of hypophysis-adrenocortex system. A persistent course of radiotherapy causes by itself and through the agency of radiotoxin it produces a hypofunction of the hematopoietic organs, the liver and the kidneys and thus lead to occurrence of radiopathic symptoms of anemia, etc.

Accordingly, for treating cases of such radiation disturbances, measures should be taken first to assure relief of disturbance in the autonomic nerve center and at the same time to relieve the patient from radiation stress, for fighting the radiation sickness in the early stage, and attention should be directed to the recovery of normal function of the affected organs in the more advanced stages.

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A New Entity of Antibody Deficiency Syndrome, to Which "Congenital Dysgammaglobulinemia" Might Be Suggested

By

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(Received for publication, March 1, 1961)

Recently we experienced a case which was clinically similar to agammaglobulinemia[1] or fatal granulomatous disease[2] but definitely different from them in laboratory findings. It was a new entity of antibody deficiency syndrome, to which we may as well give the simple name: "Congenital Dysgammaglobulinemia."

CASE REPORT

H.K., a boy, was born on October 9, 1957, and of an uneventful, full-term pregnancy and spontaneous delivery. The birth weight was 3,008 g. The parents and siblings were healthy. Small-pox and diphtheria vaccinations were performed at the age from 2 to 3 months. The latter resulted in normal primary response. Since affected by pneumonia at four months of age, the child had frequently suffered from upper respiratory tract infections or bronchitis with delayed recovery though he was treated with antibiotics each time. With the chief complaints of hepatosplenomegaly and enlargement of generalized lymph nodes, the child was admitted to our Hospital at the age of one year and two months. Blood examination at that time revealed no abnormality. The liver and kidney functions were within normal limits. Under an inconclusive diagnosis, the child's condition was slightly improved by the treatment with antibiotics and the patient was discharged after hospitalization of about one month. Respiratory infections, however, were as frequently repeated as ever after leaving hospital. Both hepatosplenomegaly and enlargement of lymph nodes tended to gradual increase again. The child was readmitted to our Hospital at the age of 2 years and 9 months for a medical re-examination. Physical examinations, on the second admission, showed a poorly nourished, pale boy with hepatosplenomegaly and generalized enlargement of lymph nodes. The abdomen was distended without fluctuation or varicosis. The non-tender liver

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with smooth surface was palpable 2 cm below the costal margin on the right mid-clavicular line and the spleen was palpable 7.5 cm below the left costal margin. The retroauricular lymph nodes were enlarged to 2×2.5 cm in diameter, several cervical lymph nodes to 0.5×0.5 cm, supraclavicular lymph nodes to 0.5×1.0 cm and axillar lymph nodes to 3×2 cm on both sides, respectively. These lymphadenopathic nodes were non-tender. No petechia or ecchymosis was present. There was no remarkable findings on the physical examination of the chest.

Table I. Blood Picture of Our Own Case

1960		Jul. 23	Aug. 10	Sep. 8	Oct. 12
R.B.C. 10^4/mm^3		210	203	349	475
Hb. g/dl.		5.9	6.5	10.5	12.8
Ht. %		21	20		41
C.I.		0.94	1.07	1.00	0.88
V.I.		1.11	1.10		0.96
Reticul. ‰		12			6
W.B.C.		6300	4600	8400	13000
Differential	B. %	0	0	0	0
	E. %	6	6	0	3
	L. %	49	49	31	55
	N. %	39	34	68	41
	M. %	6	11	1	1

Laboratory data :— Blood examination disclosed a normocytic, normochromic anemia without leukemic cells or any other abnormal cells (Cf. Table I). The bone marrow showed an erythrocytic hyperplasia, indicating 0.89 of G : E-index, a decrease of plasma cells (0.6%), and the absence of leukemic cells or other abnormal cells such as Gaucher's cells. The bleeding time was 4 minutes and the coagulation time was 6 minutes. BSR on three occasions was increased. Wassermann's reaction was negative. The fasting blood sugar was 92 mg per 100 ml. The urine was negative for protein and sugar. Bence-Jones protein test in urine was negative on several occasions. The liver function tests, including thymol turbidity test, cobalt test, Takata's reaction and BSP test, were all within normal limits. E.C.G. revealed no abnormality and x-ray examinations for chest, head and extremities showed normal findings. There was no abnormality on fundoscopic examination. Cultures from throat yielded staphylococcus aureus, streptococcus viridans and pneumococcus. The electrophoretic analysis of serum protein showed a remarkable increase of gammaglobulin fraction (Cf. Table II).

Immunologic data : — During the second hospitalization, it was suspected

Table II. Electrophoretic Pattern of Serum Protein of Our Own Case

1960	Aug. 9	Aug. 19	Oct. 13	Dec. 2
Total Protein (g/dl.)	7.50	7.80	7.60	7.95
Albumin "	3.17	2.98	3.90	3.50
Globulin "	4.33	4.82	3.70	4.45
α "	0.80	0.99	0.85	0.80
β "	1.36	1.42	1.05	1.20
γ "	2.17	2.41	1.80	2.45
A/G ratio	0.73	0.62	1.05	0.79
Remarks	Before treatment, hepatosplenomegaly, lymphadenopathy and continued fever	After inoculation of typhoid and paratyphoid vaccines	On discharge, general conditions and lymphadenopathy improved after treatment	Recurrence of upper respiratory infections and bronchitis with lymphadenopathy

that the patient might possibly have an immunologic defect because of susceptibility to infections. So the following studies were carried out.

The patient was of blood group A. However, an extremely low level of β-isohemagglutinin in the serum was demonstrated (Cf. Table III). According to Ou[3], the average of β-isohemagglutinin titer was 1 : 70.2, ranging from 1 : 512 to 1 : 8, in 29 children of blood group A aged from 2 to 5 years. Therefore, the low titer demonstrated in the present case (Cf. Table III) was considered to be decidedly abnormal. The child, who had a BCG-inoculation about one year previously, was negative both for tuberculin reaction and for Middlebrook-Dubos reaction[4]. Typhoid and paratyphoid vaccins were injected to the patient twice with the interval of a week and the Widal reaction was investigated one, two, and four weeks after each vaccination and of course just before it. The results of the Widal reaction in the present case were all negative, while those obtained from

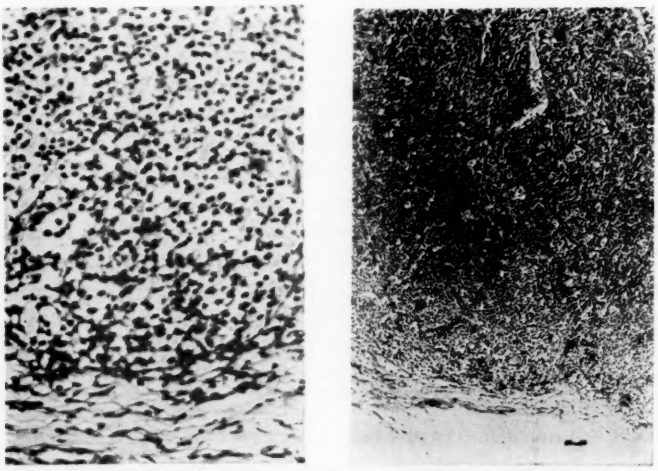
Table III. Isohemagglutinin Titer in Serum of Our Own Case

Time in Minutes \ Dilution of Sera		1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
10	P*	−	−	−	−	−	−	−	−
	C**	+	+	+	+	±	−	−	−
15	P	−	−	−	−	−	−	−	−
	C	+	+	+	+	±	−	−	−
30	P	±	−	−	−	−	−	−	−
	C	+	+	+	+	+	+	−	−

* P : Patient
** C : Control; healthy child of the same age as the patient

healthy children of the same age as the patient were positive in dilution of 1 : 50 to 1 : 100 in two and four weeks following the vaccinations. Viral antibodies against mumps, HVJ (Hemagglutinating Virus of Japan), and HA_2 (Hemadsorption Virus Type 2) were not demonstrated in the serum of the patient either. The value for C-reactive protein, which was supposed to be involved in antibody production, was always completely negative in the patient on repeated examinations, despite of the occurrence of staphylococcus, streptococcus, pneumococcus, etc. in the throat swab. Indeed each of above-mentioned results—each for itself —- might not always be a positive proof for decreased ability of antibody production. But the above results, taken in all, will mean that the patient has a defect of antibody production.

Biopsy finding of lymph nodes (Cf. Fig. 1) :— A biopsy of enlarged lymph nodes revealed a proliferation of reticulum cells and a thinning of cortex, but no evidence of malignancy such as leukemia, sarcoma, Hodgkin's disease, etc. was seen. Plasma cells were scarcely found in the sections.



(a) A remarkable proliferation of reticulum cells is noticed, but plasma cells are scarcely found.

(b) Thinning of cortex is found.

Fig. 1. Biopsy findings of a lymph node in our own case.

Clinical course :— The child ran a feverish course (37.5 to 38.5°C) and the enlarged liver and spleen and the lymph nodes swollen ahead, showed a tendency to gradual enlargement in spite of antibiotics therapy. Though a successive blood transfusion for one week was effectual for an improvement of

the anemia, it did not influence either the hepatosplenomegaly or the lymphadenopathy. Then the administration of prednisolone was begun with an initial dose of 20 mg a day, followed by gradual reduction of dose for one month (total dose : 290 mg), resulting in an improvement of general condition — drop in temperature, increase of appetite, etc. Hepatosplenomegaly and lymphadenopathy, however, showed little change. Thereafter intramuscular injections of normal human gammaglobulin (Takeda) were carried out with a view to improve the alleged antibody deficiency syndrome. A remarkable decrease in size of enlarged lymph nodes was observed by a daily injection of 0.3 g of it for successive ten days. But decrease in size of the spleen and liver took place more slowly. Then the treatment with prednisolone alone was continued without recurrence of the above symptoms and signs until the 84th hospital day when the patient was discharged under a comparatively good condition.

On discharge, the enlarged lymph nodes of all the body had decreased in size ; they were much smaller than on admission. The liver was palpable 1 cm, and the spleen was palpable 6.5 cm below the costal margin. The gammaglobulin content decreased and A/G ratio increased to a certain degree as compared with these figures on admission (Cf. Table II).

After discharge — now no medical treatment was performed, — the child had had upper respiratory tract infections and bronchitis again during one month following the discharge. During this time, the enlargement of lymph nodes had recurred, accompanied with rise of gammaglobulin content in serum (Cf. Table II). With a renewed treatment with gammaglobulin, prednisolone and antibiotics, an amelioration of the above affections and symptoms ensued.

DISCUSSION

Such a disease as the case reported here resembles one of the following diseases in symptomatology. The differential diagnosis will be discussed among them.

Fatal granulomatous disease :— This disease described by Berendes *et al.*[2] in 1957 has the following feature ; (a) frequent attacks of infections, (b) chronic lymphadenopathy which tends to suppurate gradually, (c) hepatosplenomegaly, (d) eczematoid dermatitis and blepharoconjunctivitis, (e) hypergammaglobulinemia, (f) pulmonary infiltration, (g) granulomatous nodes of the liver or lymph nodes on biopsy, (h) chronic, progressive course, resulting in death between 6 months and 3 years after the onset, (i) occurring predominantly in male occasionally with hereditary incidence, (j) fever reminding one of sepsis, and (k) anemia, sometimes with eosinophilia.

The present case had the items (a), (c), (e), (j) and (k) in common, with the disease in question, however, there was no tendency to suppuration of lymph nodes in our own case through the whole course of 1½ years. In addition, it is different from the above disorder in that lymphadenopathic nodes of our own

patient showed a decrease in size following the treatment, and in that there was no evidence of pulmonary infiltration (j) on x-ray films of the present case. A biopsy of lymph nodes of our own patient revealed no granulom but only a reactive reticulosis. On the other hand, the inability for antibody production and the defect of isohemagglutinin both of which are probably characteristics of our own case — do not occur in fatal granulomatous disease. It was, therefore, concluded that the present disease was different from fatal granulomatous disease, although both of them showed some similarities in symptomatology.

Congenital a- or hypo-gammaglobulinemia :— Since the first description by Bruton[1] in 1952, many studies[5-14] have been published concerning this disorder. The features of the disease are as follows ; (a) frequent attacks of infection beginning between 9 months and 2 years of age, (b) staphylococcus, streptococcus, pneumococcus, meningococcus, etc. as common organisms causing infections, (c) a normal primary response against vaccination in early infancy in two thirds of the patients, (d) low concentration of gammaglobulin in plasma, mostly below 50 mg per 100 ml, (e) absence or extremely low titers of isohemagglutinin in serum, (f) failure to develop antibodies against specific antigen, (g) a hyperplasia of reticulum cells, thinning of cortex and decrease of plasma cells and lymphocytes — all of which will be revealed on biopsy of lymph nodes, and (h) predominant occurrence in male and transmission by a sex-linked recessive trait.

A definite difference between this disorder and our own case lies in concentration of gammaglobulin in plasma. It is, however, curious that there is an agreement of findings between these two disorders except gammaglobulin content. Especially the case of hypogammaglobulinemia reported by Dolan *et al.*[14] was like our own patient accompanied with anemia and hepatosplenomegaly. Accordingly, it seems to be likely that increased gammaglobulin in our own case might be immunologically abnormal one, as will be discussed later.

Multiple myeloma : — In multiple myeloma, hypergammaglobulinemia is occasionally present[15] and susceptibility to recurrent infections, diminishing of antibody response to antigenic challenge and absence of isohemagglutinins have been described. Bence-Jones protein, which is inevitably present in urine of multiple myeloma, was not found in urine of the present case, in spite of repeated examinations. Multiple myeloma can further be excluded by age incidence, bone marrow picture and x-ray findings of the bone, in addition to the absence of Bence-Jones protein already mentioned.

Janeway's cases and Hallman's cases (Cf. Table IV) :— The cases reported by Janeway[16] in 1954 and the cases by Hallman[17] in 1956 were similar to our own case, too. This case, however, was different from Janeway's cases in regard to inability of antibody production and decrease of plasma cells in lymph nodes as shown in Table IV. Hallman's report is devoid of description as to ability for antibody production and isohemagglutinin.

TABLE IV. Comparison among Janeway's, Hallman's Cases and Our Own Case

	Janeway's cases	Hallman's cases	Present case
Susceptibility to infections	+	+	+
Hepatosplenomegaly	+	+	+
Lymphadenopathy	+	−	+
Anemia	?	+	+
Fever	?	+	+
Hypergammaglobulinemia	+	+	+
Biopsy of lymph nodes	mononuclear infiltration and prominence of plasma cells	mononuclear infiltration	Proliferation of reticulum cells, thinning of cortex and decrease of plasma cells
Antibody response against antigenic stimulation	normal	?	absent
Isohemagglutinin titer	No description	No descriptoin	extremely low
Effect by γ-globulin	No description	+	+

Although it is generally accepted that gammaglobulin represents the major parts of antibody, it remains yet uncertain whether or not all of gammaglobulin is antibody. It may be reasonable to presume that an elevation of gammaglobulin demonstrated in the present case is a reflection of immune responses to recurrent infections. Nevertheless, an extremely poor response to antigenic stimulation (see p. 301), just as observed in agammaglobulinemia, was demonstrated in the present case. The fact that a small-pox vaccination in early infancy resulted in normal primary response in the present case might be due to the normal gammaglobulin which had been derived from mother and remained to stay during his early infancy. This could be explained on the basis of half life of gammaglobulin[18-20]. An inference may be drawn from the above-mentioned results that the increased gammaglobulin in question in our own case would not be an antibody gammaglobulin but rather be a nonspecific, nonantibody gammaglobulin. It is, therefore, possible to presume that there was a disturbance in productive process of antibody gammaglobulin in the reticuloendothelial system, a site of gammaglobulin production, in the present case, causing an overproduction — a futile overproduction of nonantibody gammaglobulin. The finding that no antibody production took place against specific antigen while increase of gammaglobulin was observed in the present case (Cf. Table II) will probably support the above inference. Though the present case, from viewpoint of gammaglobulin content in serum, is quite different from a case of agammaglobulinemia, both disorders seem to have an immunological similarity in that both are unable to develop antibody. Since plasma cell is thought to be closely associated

with production of antibody gammaglobulin[21-27], hypergammaglobulinemia ought to be usually accompanied with increase of plasma cells in the reticuloendothelial system. In the present case, however, there was no expected proliferation of plasma cells either in lymph nodes or bone marrow; on the contrary, a more or less striking decrease of these cells was seen (Cf. Fig. 1). It is a question that nonantibody gammaglobulin — in contrast to antibody gammaglobulin — has been produced by plasma cells in the present case. Grant[28] stated that splenomegaly in agammaglobulinemia might be due to a reactive hyperplasia of the spleen which is probably one of sites of gammaglobulin formation. Then it may not be too much to say that also in our own case of deficiency of antibody gammaglobulin hepatosplenomegaly and lymphadenopathy occurred by way of compensation.

Now basing upon the above-mentioned presumption that the present case might have a disturbance in productive process of antibody gammaglobulin, we should like to suggest the term "Congenital Dysgammaglobulinemia" for this particular entity of antibody deficiency syndrome in contrast to the syndrome of agammaglobulinemia. The relationship between agammaglobulinemia and "dysgammaglobulinemia" is illustrated schematically as shown in Fig. 2 (Cf. Fig. 2). In normal individuals, antigen due to infection stimulates the reticuloendothelial system to produce both antibody gammaglobulin and nonantibody

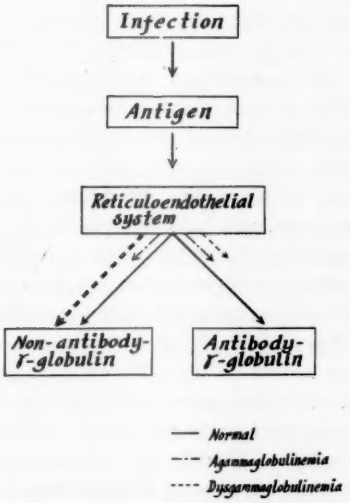


Fig. 2. Schematic illustration of dysgammaglobulinemia and agammaglobulinemia.

gammaglobulin, only the former playing a role of prevention from infection. In agammaglobulinemia production of both antibody gammaglobulin and nonantibody gammaglobulin might be affected. On the other hand, the production of antibody gammaglobulin alone was probably affected in "dysgammaglobulinemia", resulting in overproduction of nonantibody gammaglobulin and susceptibility to infection. It might be due to its inhibitory action upon overproduction of nonantibody gammaglobulin that prednisolone was effective in our own case. The remarkable effect induced by normal gammaglobulin in the present case might be explained in such a way that normal gammaglobulin, induced by injection, alleviated the stimulated reticuloendothelial system, resulting in inhibition from overproduction of nonantibody gammaglobulin.

The ultracentrifugal analysis of serum protein in the present case indicates the results as shown in Table V (Cf. Table V). The molecular weights of A-component and G-component were within normal limits, and no macroglobulin or cryoglobulin was noticed. An increase of G-component may be a reflexion of elevated gammaglobulin. M-fraction, which should show approximately only 2%

Table V. Ultracentrifugal Analysis of Serum Protein

Component	Sedimentation constant	Molecular weight	Percentage in quantity
A	3.9	43,000	48 %
G	6.7	87,000	40 %
M	15.0	250,000	12 %

in normal, increased markedly. Although the significance of M-component is difficult to understand, it is probable that the increase of this component in the present case might be accompanied with a disorder of gammaglobulin production in the reticuloendothelial system. Smith[29] described that there was no significant difference in amino acid composition between normal gammaglobulin and antibody. It may be impossible to distinguish nonantibody gammaglobulin from antibody gammaglobulin by the difference of the molecular weights, since the difference between both gammaglobulin is thought to exist in a derangement of molecular structure. Since the mechanism or site involved in antibody production is not yet fully understood at present, many questions concerning same remain to be solved.

SUMMARY

A case characterized by susceptibility to recurrent infections, hepatosplenomegaly and lymphadenopathy, was described in detail. Examination revealed an increased level of gammaglobulin in serum, in spite of an extremely poor capacity for antibody production. The term "Congenital Dysgammaglo-

bulinemia", — a possible disturbance in productive process of antibody gamma-globulin in the reticuloendothelial system, — was applied to this probably new entity of antibody deficiency syndrome.

Acknowledgement

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On the Essential Nature of Hematopoietic Function of Bone Marrow

Report 1. On the Monophasis of Mitosis in the Erythropoietic System and the Diphasis of Mitosis in the Leucopoietic System in the Bone Marrow

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PREFACE

The author has found that the essential difference between leucocytosis and leucopenia originates from the diphasis of mitosis in the leucopoietic system in the bone marrow[1-15].

On the basis of this result, the principle of hematopoiesis in the bone marrow will be discussed in these reports.

1. In the ordinary clinic, the cases in which leucocytosis appears are as follows:

1) Coccal and bacillary infections: pneumonia, bronchopneumonia, Asiatic cholera, bronchitis, meningitis epidemica, erysipelas, scarlet fever, sepsis, gonorrhoea, tonsillitis, lymphadenitis submaxillaris, otitis media, liver abscess, appendicitis, puerperal fever, dysentery, pertussis, diphtheria and other infectious and purulent diseases; spirochetosis: syphilis, relapsing fever, Weil's disease; rickettsial diseases: eruptive typhus; viral diseases: various kinds of encephalitis, lyssa, small pox and other infectious and purulent diseases.

2) Chemical agents and drugs.

a) sodium nucleinate and various kinds of acid, potassium chlorate, chrome, phenylhydrazine; antipyretics, i.e. acetanilide, phenacetin, etc.; pyridine, epinephrine, turpentine, camphor, digitalis, pyrogallol.

b) Metabolic products: in uremia, diabetic acidosis, eclampsia, gout and burns and so on.

3) Acute hemorrhage.

4) Postoperatively.

5) Non-inflammatory conditions, such as coronary thrombosis.

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6) Malignant neoplasms.

7) Hemolysis of erythrocytes.

8) Physiological cases: in newborn children, during labor, after repeated vomiting and convulsions.

2. Leucopenia appears in the following cases:

1) Bacterial infectious diseases: typhoid and paratyphoid fevers, Bang's disease, Malta-fever; rickettsiosis: Japanese river fever, Ezô-fever, Shichitô-fever; viral diseases: measles, rubeola, poliomyelitis, influenza, hepatitis epidemica, mumps, dengue, Pappataci-fever; protozoal infection: Kala-azar.

2) Physico-chemical agents:

i) Physical agents: radium, Roentgen-rays, thorium X, ionising radiation, etc.

ii) Chemical agents: benzol and its derivatives, e.g. anilin, amidopyrine, dinitrophenol, sulfon-amides, etc.; gold, arsenics, sarvarsan; antithyroid drugs, e.g., thyouracil; anticancerous drugs: urethane, nitrogen mustards, nitromin, azaguanin, carcinophyllin; antimicrobial agents, e.g. chloramphenicol, antihistaminics.

3) Cachectic and debilitated states and inanition.

4) Disturbances of the liver, e.g. cirrhosis of liver; collagen diseases: Felty's syndrome, lupus erythematosus.

5) Hematopoietic disorders involving the spleen: Banti's disease, Gaucher's disease, pernicious anemia, agranulocytosis, aplastic anemia, aleukemia.

6) In anaphylactoid shock and in early stages of reaction to foreign protein.

Concerning the causative mechanism of leucocytosis and leucopenia many opinions have been uttered, while at the present time Naegeli's[16] opinion is generally accepted. He regarded leucocytosis as an expression of sufficiency and leucopenia as that of insufficiency of the bone marrow.

In the studies of the principle of hematopoiesis in the bone marrow, the most important points to which particular attention should be paid are that 1) the bone marrow should be stimulated by a natural factor without artificial procedure so that they might be allowed to take their own natural courses, and that 2) the bone marrow function should be observed by systematic serial biopsies.

The stimulant factors to the bone marrow were only pathogenetic microbes in former times, but recently, by wonderful progress of physics and chemistry many physico-chemical agents were added thereto: that is, benzol was discovered by Farady[17] in 1825; X-rays, by Röntgen[18] in 1895; uranium, by Becquerel[19] in 1896 and radium and thorium, by P. & M. Curie[20] in 1898.

Nowadays these physico-chemical agents are of great efficacy in making diagnosis as well as in treatments in medical practice. On the other hand, however, their side-effects upon the viscerae, especially upon the bone marrow have

come into question. In addition, atom-bomb was invented in 1945, whose terrible influence upon the bone marrow and sexual organs has called world-wide attention.

Yet the phylogenesis of Homosapiens in 50 myriad years has been the history of their struggle for survival against pathogenetic microbes, and the hematopoietic function in the bone marrow is an expression of an adaptation which is also attained in the course of struggle of mankind against them. Therefore, the principle of hematopoiesis in the bone marrow can be found in studying the course of infectious diseases.

Hematopoietic Function of the Bone Marrow

The bone marrow function was studied here in the course of infectious diseases allowed to take their own natural course, no antibiotics but only bone marrow puncture being applied.

The hematopoietic function of the bone marrow is divided into the formation of cells and their maturation; the former is related with the quantity of blood cells, the latter with their quality.

The appearance of basophilic stippling and anisocytosis of the erythrocytes, and those of Döhle's inclusion bodies and toxic granules of the leucocytes are regarded as due to the disturbance of the maturation function, but as it was, these findings are of little significance unless the bone marrow falls into insufficiency. On the contrary, the cell-formation function of the bone marrow is of great importance, both in hematology and in sero-immunology.

In general, the formation of blood cells depends upon mitosis. The mitosis of nucleated cells in the bone marrow was observed with the aid of Dr. Miyamoto after Rohr's[21] counting method and the mitosis index (M.I.) was calculated by following formula:

$$\text{Mitosis index } (‰) = \frac{\text{mitosis count}}{\text{nucleated cell count}} \times 1{,}000$$

1. Monophasis of Mitosis in the Erythropoietic System in the Bone Marrow

1) Mitosis in the erythropoietic system in the bone marrow of 5 normal subjects: the mitosis index of nucleated cells in the erythropoietic system is

Table I. Mitosis Index in Normal Cases

Kind of Cell	Erythropoiesis			Leucopoiesis				
	Erythroblasts			Myelo-blasts	Neutrophil myelocytes			Eosino-phils
	Baso-phil	Poly-chroma-tic	Oxyphil		Un-matured	Half-matured	Matured	
Cell counts	1660	7710	5460	670	1560	2990	4740	2450
Mitosis counts	155	304	162	2	13	55	33	7
M.I. ‰	93.3	39.3	29.2	2.99	8.33	18.39	6.98	2.86

Table II. Frequency of

Erythropoiesis				Leucopoiesis	
M.I. ‰	Erythroblasts			M.I. ‰	Myeloblasts %
	Basophil %	Polychromatic %	Oxyphil %		
38.5~46.3	18.6-33.2	42.1-53.9	12.9-39.3	2.2~2.7	0-4.3
41.84	24.04	48.44	27.52	2.40	1.60

41.84‰, and the highest one is 93.3‰ in basophil erythroblasts. 48.44% in polychromatic erythroblasts indicates the highest frequency of mitosis, as shown in Tables I and II.

Table III shows the frequency of cells in the four phases in mitosis; the highest is 54.4% in prophase, followed by metaphase, telophase, anaphase in descending order.

Table III. Frequency of Every Mitosis-Phase in Normal Cases

System	Erythropoietic system				Leucopoietic system			
Phase	Pro.	Meta.	Ana.	Telo.	Pro.	Meta.	Ana.	Telo.
%	46.6-67.6	8.8-22.9	9.1-14.3	11.8-18.2	48.2-66.6	12.5-25.8	4.6-14.8	8.4-18.2
Mean value %	54.42	18.66	12.12	14.80	55.12	20.66	10.68	13.52

2) Mitosis in the erythropoietic system in the bone marrow in leucocytosis.

In 5 cases of leucocytosis (4 cases of Japanese encephalitis and 1 case of scarlet fever), the mitosis index in the erythropoietic system in the bone marrow in every stage of illness was observed. The mitosis index, as shown in Table IV, was 41.46‰ in the acme; 42.45‰ in the fever-abating stage and 42.20‰ in the reconvalescence. In these cases, the erythrocyte count in the peripheral blood is 4.52 millions in the acme; 4.59 millions in the fever-abating stage and 4.78 millions in the reconvalescence. That is to say, unless anemia appeared in the peripheral

Table IV. Relation between Mitosis Index in the Erythropoietic System and the Red Blood Cell Count in the Peripheral Blood in Every Stage of the Illness

Disease	Leucocytosis			Leucopenia		
Stage of the illness	Acme	Fever-abating	Reconv.	Acme	Fever-abating	Reconv.
M.I. ‰	38.7-47.0	4.02-44.2	38.2-47.2	35.9-48.4	35.5-50.5	38.6-58.1
Mean value ‰	41.46	42.45	42.20	42.28	42.74	45.88
R.B.C. (Million)	3.53-5.44	3.88-5.39	4.00-5.31	2.64-4.15	2.98-4.48	3.66-4.72
Mean value (Million)	4.52	4.59	4.78	3.35	3.85	4.33

Mitosis in Normal Cases

Leucopoiesis				
Neutrophil myelocytes			Meta-myelocytes %	Eosinophils %
Unmatured %	Half-matured %	Matured %		
4.6-16.6	39.2-58.2	25.0-54.6	0	0-13.1
10.80	45.74	36.08	0	5.78

blood, the mitosis index in erythropoietic system in the bone marrow showed nearly 42‰ of its normal value. Table V shows the mitosis index in various kinds of erythroblasts in every stage of illness; the mitosis index showed the highest value of 52.7‰ in polychromatic erythroblasts in the acme, and returned to the normal value after the fever-abating stage.

Table V. Mitosis Index in Various Kinds of Cells in the Erythropoietic System in Every Stage of the Illness

Disease		Leucocytosis			Leucopenia		
Stage	Kind of Cell	Basophil	Polychromatic	Oxyphil	Basophil	Polychromatic	Oxyphil
Acme	Cell counts	1,187	3,770	3,602	1,036	2,740	5,108
	Mitosis counts	62	119	90	93	137	130
	M.I. ‰	52.3	52.7	24.9	89.7	50.0	25.6
Fever-abating	Cell counts	578	2,294	1,238	1,456	3,710	3,912
	Mitosis counts	34	102	38	108	192	144
	M.I. ‰	59.0	47.2	30.8	74.1	54.4	29.3
Reconvalescence	Cell counts	1,374	3,396	4,310	1,139	3,740	4,368
	Mitosis counts	79	187	113	92	214	136
	M.I. ‰	57.4	55.2	62.2	80.7	57.2	31.0

In respect of the frequency of mitosis in every stage of the illness, polychromatic erythroblasts occupied the highest position throughout the entire course. And the frequency of four phases of mitosis in the erythropoietic system was similar to that in normal subjects, as shown in Table VI.

Table VI. Frequency of Motisis in the Erythropoietic System in Every Stage of the Illness (%)

Disease	Leucocytosis			Leucopenia		
Kind of Cell / Stage	Basophil	Polychromatic	Oxyphil	Basophil	Polychromatic	Oxyphil
Acme	17.8	56.6	25.6	25.8	38.1	36.1
Fever-abating	19.5	58.7	21.8	24.4	43.4	32.2
Reconvalescence	20.8	49.4	29.8	20.8	48.5	30.7

3) Mitosis in the erythropoietic system in the bone marrow in leucopenia.

In 5 cases of leucopenia (3 cases of typhoid and 2 cases of paratyphoid A), mitosis index in the erythropoietic system in the bone marrow was observed: the mitosis index was 42.28‰ in the acme; 42.74‰ in the fever-abating stage and 45.88‰ in the reconvalescence, as shown in Table IV. At the same time, the erythrocyte count amounted to 3.35 millions in the acme stage; to 3.85 millions in the fever-abating stage and to 4.33 millions in the reconvalescent stage. These are significant in showing that the mitosis index was hightened in the course of illness when anemia appeared in the peripheral blood.

The mitosis index showed the highest value of 89.7‰ in basophil erythroblasts through the entire course of illness, as shown in Table V. And the frequency of mitosis in polychromatic erythroblasts showed the highest percentage in every stage of the illness (Table VI). Among the four phases of mitosis in the erythropoietic system, the prophase showed the highest frequency through the entire course, and the frequency was similar to that seen in normal subjects (Table VII).

TABLE VII. Frequency of Every Mitosis-Phase in the Erythropoietic System (%)

Disease	Leucocytosis				Leucopenia			
Phase	Pro.	Meta.	Ana.	Telo.	Pro.	Meta.	Ana.	Telo.
Acme	39.2	19.1	13.5	28.2	37.7	23.9	14.3	24.1
Fever-abating	41.6	25.3	12.6	20.5	44.6	25.2	12.6	17.6
Reconvalescence	39.1	20.3	14.0	26.6	44.4	21.2	14.5	19.9

As observed above, the mitosis in the erythropoietic system in the bone marrow shows nothing characteristic either in leucocytosis or leucopenia, as compared with that in normal subjects. But, when anemia occurs in the peripheral blood, CO_2 concentration is elevated in the body and the latter acts as a stimulant factor upon the bone marrow, raising mitosis in the erythropoietic system in the bone marrow. The rise of mitosis is at first on the left, later gradually on the right in the bone marrow and the erythrocytes increase gradually to the normal value. Therefore, it can be recognized that the erythrocytes are formed in the monophasis in the bone marrow, so long as it is insufficiency.

2. Diphasis of Mitosis in the Leucopoietic System in the Bone Marrow

A. 2 trends of mitosis in the leucopoietic system in the bone marrow.

1) Mitosis in the leucopoietic system in the bone marrow of normal subjects.

In the leucopoietic system in the bone marrow of above-mentioned 5

normal subjects, the mitosis index of the nucleated cells is 2.4‰. The mitosis index of the half-matured myelocytes shows the highest value of 18.39‰ (Table I) and the frequency of mitosis of the half-matured myelocytes the highest of 45.74% (Table II).

The frequency of the four phases of mitosis, as shown in Table III, rises to the highest value of 55.12% in the prophase and next in the metaphase, telophase and anaphase in the order named.

2) Mitosis in the leucopoietic system in the bone marrow in leucocytosis.

In the 5 cases of leucocytosis above mentioned, the mitosis index in the leucopoietic system amounted to 3.28‰ in the peak stage, being apparently more heightened than in normal cases. It is obviously lowered to the somewhat subnormal height of 2.30‰ in the fever-abating stage, but rose again in the reconvalescence, showing almost normal value of 2.58‰. On the other hand, the leucocyte count in peripheral blood was 11,440 in the peak stage, 9,400 in the fever abating stage and 8,360 in the reconvalescence (Table VIII).

Table VIII. Relation between the Mitosis Index in the Leucopoietic System and the White Blood Cell Count in the Peripheral Blood in Every Stage of the Illness

Disease	Leucocytosis			Leucopenia		
Stage	Acme	Fever-abating	Reconv.	Acme	Fever-abating	Reconv.
M.I. ‰	2.6-4.3	1.8-3.2	1.8-3.3	1.6-2.4	1.7-3.8	2.1-2.4
Mean value ‰	3.28	2.30	2.58	1.94	2.52	2.26
W.B.C.	9,400-13,300	7,400-12,000	7,000-9,700	2,600-7,000	2,500-6,300	3,300-6,800
Mean value	11,440	9,400	8,360	4,000	4,840	5,540

Table IX shows the mitosis index in various kinds of the nucleated cells in the leucopoietic system in the bone marrow; mitosis is strikingly accelerated in various kinds of cells and the mitosis index of matured myelocytes rises remarkably in the peak stage, but thereafter mitosis gradually slows down and the mitosis index of unmatured myelocytes shows the highest value in the course of illness as in normal subjects. The frequency of mitosis in every stage of the illness, as shown in Table X, shows the highest percentage of 43.0% in the matured myelocytes and in the later course of the illness, the center of mitosis, shifting from right to left in the bone marrow, returned to the normal.

In the frequency of four phases of mitosis in the leucopoietic system, no great difference is there between leucocytosis cases and normal cases, as shown in Table XI.

3) Mitosis in the leucopoietic system in the bone marrow of leucopenia.

In the 5 cases of leucopenia mentioned above, the mitosis index in the

Table IX. Mitosis Index in Various Kinds of Cells in

Disease		Leucocytosis			
Stage	Kind of cell	Myeloblasts	Myelocytes		
			Unmatured	Half-matured	Matured
Acme	Cell counts	607	1,175	2,152	4,227
	M. counts	9	23	58	71
	M.I. ‰	14.8	19.6	26.9	16.9
Fever-abating	Cell counts	378	914	1,690	2,478
	M. counts	2	14	32	30
	M.I. ‰	5.4	15.2	19.0	12.2
Reconva-lescence	Cell counts	773	1,215	2,294	4,079
	M. counts	5	25	59	43
	M.I. ‰	6.5	20.5	25.8	10.5

Table X. Frequency of Mitosis in the Leucopoietic System

Kind of Cell	Leucocytosis					
	Myelo-blasts	Myelocytes			Metamyelo-cytes	Eosinophils
		Unmatured	Half-matured	Matured		
Acme	5.4	14.0	35.2	43.0	0	2.4
Fever-abating	2.2	15.6	35.6	33.3	0	13.3
Reconva-lescence	3.9	15.9	37.6	27.4	0	15.9

Table XI. Frequency of Every Mitosis-Phase in the Leucopoietic System (%)

Disease	Leucocytosis				Leucopenia			
Phase	Proc.	Meta.	Ana.	Telo.	Pro.	Meta.	Ana.	Telo.
Acme	57.6	17.0	17.5	7.9	22.5	42.8	27.6	7.1
Fever-abating	55.5	6.7	6.7	17.8	38.7	33.9	16.2	11.2
Reconvalescence	51.7	15.3	15.3	8.9	57.2	25.0	14.3	3.5

leucopoietic system was 1.94‰ in the peak stage, 2.52‰ in the fever-abating stage and 2.26‰ in the reconvalescence, as presented in Table VIII. On the other hand, the leucocyte count in the peripheral blood is 4400 in the peak stage, 4840 in the fever-abating stage and 5540 in the reconvalescence. Table IX shows the mitosis index of various kinds of the nucleated cells in the leucopoietic system in every stage of the illness; the mitosis index was lowered in the half-matured myelocytes, particularly in the matured myelocytes in the

the Leucopoietic System in Every Stage of the Illness

Eosinophils	Leucopenia				
	Myeloblasts	Myelocytes			Eosinophils
		Unmatured	Half-matured	Matured	
2,602	580	853	1,687	4,118	1,389
4	5	17	35	37	4
1.5	8.6	19.9	20.6	9.1	2.8
1,592	428	1,242	1,708	3,978	992
12	6	20	50	44	4
7.6	14.0	16.0	29.2	11.0	4.0
6,111	446	932	1,636	3,526	1,266
25	4	30	50	40	4
4.1	9.0	32.2	30.6	12.6	3.2

in Every Stage of the Illness (%)

Leucopenia					
Myeloblasts	Myelocytes			Metamyelo-cytes	Eosinophils
	Unmatured	Half-matured	Matured		
5.6	17.4	35.7	37.8	0	3.5
4.8	16.2	40.3	35.4	0	3.3
1.7	23.2	39.3	32.2	0	3.6

peak stage, but after the fever-abating stage rose gradually from left to right in the bone marrow and then returned to normal. The frequency of mitosis showed the highest percentage of 37.8% in matured myelocytes in the peak stage; of 40.3% in half-matured myelocytes in the fever-abating stage, as shown in Table X.

The frequency of four phases of mitosis showed the highest percentage in the metaphase in the peak stage, but after the fever-abating stage its highest percentage was shown in the prophase, as in normal subjects (Table XI). Table

TABLE XII. Relation between Mitosis Index and the White Blood Cell Count in the Peripheral Blood

W.B.C.	2,001-6,000	6,001-9,000	9,001-13,000
Disease	Leucopenia	Normal	Leucocytosis
M.I. ‰	1.90-2.38	2.20-3.10	2.60-4.30
Mean value ‰	2.17	2.50	3.20

XII shows the relation between mitosis index in the leucopoietic system in the bone marrow and the leucocyte count in the peripheral blood.

4) Mitosis in the Reticuloendothelial System in the Bone Marrow.

Throughout the entire course of leucocytosis, the mitosis index in the reticuloendothelial system rose to the height of 2.16‰ in the peak stage showing a supernormal value, but in leucopenia showed almost normal value of 1‰ (Table XIII).

TABLE XIII. Mitosis Index in the Reticuloendothelial System in Every Stage of the Illness

Disease	Leucocytosis			Leucopenia		
Stage of the illness	Acme	Fever-abating	Reconv.	Acme	Fever-abating	Reconv.
M.I. ‰	0-4.5	0-3.2	0-3.5	0-2.0	0-2.9	0-2.4
Mean value ‰	2.16	1.32	1.45	1.08	0.98	1.24

Rising or lowering of mitosis in the bone marrow shows increase or decrease of the nucleated cells in the bone marrow; that is to say, the bone marrow was rich in nucleated cells in the cases of leucocytosis, while it was poor in them in the cases of leucopenia (Tables XIV and XV).

Accordingly, the nucleated cell count in the former not rarely reached the level almost twice or thrice as high as in the latter. Thus, the author has found that the mitosis index tends toward rising in the cases of leucocytosis, while in the cases of leucopenia tends toward lowering, the mitosis index attaining the normal level after the reconvalescence in both cases.

B. Diphasis of mitosis in the leucopoietic system in the bone marrow.

The author inquired into the two trends of mitosis in the leucopoietic system in the bone marrow. According to the leucocyte counts of the cases, all the infectious diseases can be divided into two groups: those accompanying leucocytosis and those with leucopenia. From the angle of genetic mechanism, the difference between the two originates in their mitotic function in the bone marrow; namely, it is plainly raised in leucocytosis, while it is plainly lowered in leucopenia.

The values of mitosis index in every stage of leucocytosis and leucopenia are marked with point co-ordinates, setting the axis of the co-ordinates at the level of 2.4‰ of normal mitosis index: 3.28‰ in the acme, 2.30‰ in the fever-abating stage and 2.58‰ in the reconvalescence of leucocytosis; in the same way, 1.94‰ in the acme, 2.52‰ in the fever-abating stage and 2.26‰ in the reconvalescence of leucopenia. When traced successively, these points present a pair of wavy curves shown in thick lines in Fig. 1.

When these curves are corrected on the basis of outflows of leucocytes into

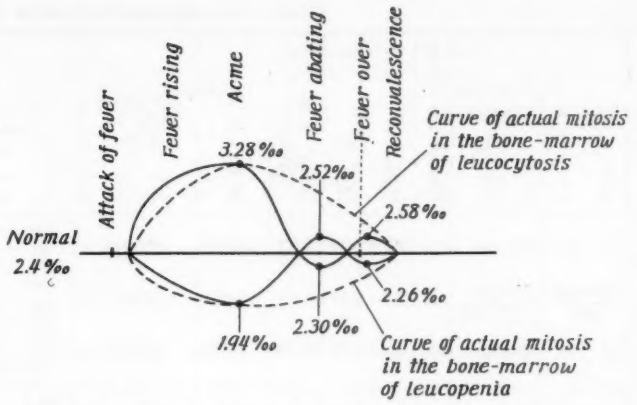

Fig. 1. Curves of mitosis index of leucopoietic system in the bone marrow in the course of leucocytosis and leucopenia.

the peripheral blood and on the basis of changes of the nucleated cell count in the bone marrow, they can be shown as a pair of simple symmetric curves (dotted curves) with trends toward rising and lowering respectively, and they indicate the actual mitosis in the leucopoietic system in the bone marrow of leucocytosis and leucopenia.

After six years of contemplation of these experimental results, the conception of phases was at last acquired. If the pair of these curves be taken to represent the 2 trends in one and the same phase, the downward trend is naturally to be considered as showing an insufficiency of the bone marrow; while on the contrary, if it be taken to show the 2 peculiar trends in 2 phases respectively, then the downward trending is to be considered not as indicating an insufficiency but a sufficiency of the bone marrow, namely, a peculiar trend of mitosis which appears when the bone marrow is stimulated.

Which of the two interpretation of the pair of these curves is the right one? Seeing the specificity of pathogenetic factors described below, it is clear that the latter is the right one.

Here the author has named the upward mitotic course the first phase, and the downward mitotic course the second phase; also the factors active in the former the first-phase factors, and these in the latter the second-phase factors. Accordingly, the specificity among the factors not merely in the different phases but also in the same phase can be recognized regularly and definitely.

Thus both in the first- and second- phase the normal bone marrow is stimulated by the pathogenetic microbes, and reacts physicologically against them according to the specificities of their factors.

It is, therefore, improper simply to take increase of the white blood cells

TABLE XIV. Frequency of Every Phase

Name Age Sex	Disease	Stage	Day of the illness	Temp. °C	Hgb %	R.B.C. in B. (Million)	W.B.C. in B.	Nucleated cells in B.M.	Counted cells
E. Y. 18 years old ♂	Enceph. jap.	Acme	7	39.9	72	3.53	12200	154000	Ret 526 R 2134 W 10000
		Fever-abating	11	37.4	81	4.21	9000	199000	Ret 286 R 1070 W 10000
		Reconv.	25	36.8	83	4.00	9700	237000	Ret 382 R 2220 W 10000
M. Y. 5Y. 10M. ♂	Enceph. jap.	Fever-abating	16	37.4	80	3.88	7400	351200	Ret 301 R 794 W 10000
		Reconv.	25	37.1	86	4.89	7000	181000	Ret 280 R 1966 W 10000
		Reconv.	39	36.5	93	5.31	9500	298000	Ret 402 R 1544 W 10000
S. K. 8 J years old ♂	Enceph. jap.	Acme	7	38.6	95	5.44	13300	91000	Ret 437 R 817 W 10000
		Fever-abating	14	38.0	92	5.39	9200	99300	Ret 594 R 890 W 10000
		Reconv.	26	37.1	89	4.95	8800	142300	Ret 866 R 1430 W 10000
S. T. 19 years old ♀	Enceph. jap.	Acme	12	38.1	90	4.75	9400	138400	Ret 588 R 2004 W 10000
		Acme	41	37.9	90	4.15	12200	162000	Ret 690 R 2326 W 10000
		Fever-abating	55	37.6	89	4.89	12000	127400	Ret 472 R 1356 W 10000
S. T. 6 years old ♀	Scarlet fever	Acme	2	38.9	91	4.73	10100	125000	Ret 478 R 1278 W 10000
		Reconv.	9	36.7	89	4.68	8200	157000	Ret 406 R 1412 W 10000
		Reconv.	16	36.4	87	4.84	8000	88000	Ret 360 R 508 W 10000

of the Mitosis in Leucocytosis

M. counts	M.I. %	Appearance of mitosis															
		Erythropoiesis							Leucopoiesis								
		Baso.	Polychr.	Oxy.	Pro.	Meta.	Ana.	Telo.	Mbl.	Unm. M.	Half-m. M.	Mat. M.	Meta-M.	Eos.	Pro.	Meta.	Ana.	Telo.
0	0																
84	39.3	20	52	12	34	24	12	14	2	4	10	8	0	2	16	6	4	0
26	2.6																
0	0																
42	42.3	12	22	8	20	8	6	8	0	6	10	10	0	6	20	6	0	6
32	3.2																
0	0																
92	41.6	14	40	38	36	24	12	20	0	6	12	8	0	8	16	8	6	4
33	3.3																
0	0																
34	42.7	10	18	6	14	12	4	4	0	2	6	8	0	2	10	4	0	4
18	1.8																
1	3.5																
86	43.7	24	46	16	30	20	8	28	2	4	8	4	0	6	12	6	4	2
24	2.4																
1	2.5																
66	42.7	16	32	18	28	16	10	12	2	4	10	10	0	8	20	8	6	0
33	3.3																
2	4.5																
33	40.4	2	13	18	20	3	3	7	1	5	10	11	0	0	13	6	5	3
27	2.7																
2	3.2																
38	42.7	4	24	10	16	12	4	6	0	2	8	8	0	0	8	4	4	2
19	1.9																
0	0																
57	39.8	11	29	17	20	6	11	20	1	3	13	5	0	3	13	8	2	2
25	2.5																
2	3.4																
84	41.9	12	46	26	18	12	18	36	4	4	12	20	0	0	22	8	6	4
39	3.9																
2	2.9																
90	38.7	20	52	18	40	20	8	22	2	2	14	8	0	2	16	4	6	2
29	2.9																
1	2.1																
60	44.2	8	38	14	22	12	8	18	2	4	8	4	0	4	12	4	2	4
23	2.3																
0	0																
67	47.0	8	36	16	24	10	6	20	0	8	12	24	0	0	28	4	8	4
43	4.3																
0	0																
54	38.2	8	28	18	26	6	6	16	0	4	6	8	0	0	8	4	4	2
18	1.8																
1	2.7																
24	37.2	6	12	6	8	6	6	4	0	4	10	8	0	0	12	4	2	4
22	2.2																

TABLE XV. Frequency of Every Phase

Name Age Sex	Disease	Stage	Day of the illness	Temp. °C	Hgb. %	R.B.C. Mill.	W.B.C. in B.	Nucleated cells in B. M.	Counted cells	
S. S. 27 years old ♀	Para-typhoid A	Acme	6	38.9	74	3.12	4000	112000	Ret	674
									R	536
									W	10000
		Fever-abating	27	37.1	78	3.68	5400	105000	Ret	362
									R	1072
									W	10000
		Reconv.	41	36.7	70	3.66	6200	95000	Ret	642
									R	1902
									W	10000
M. M. 25 years old ♀	Para-typhoid A	Acme	12	38.8	73	3.70	5200	180000	Ret	1592
									R	1956
									W	10000
		Fever-abating	26	38.2	79	3.89	5200	135000	Ret	988
									R	1942
									W	10000
		Reconv.	33	36.3	81	4.14	5600	144000	Ret	652
									R	2582
									W	10000
H. Y. 25 years old ♂	Typhoid	Fever-abating	14	37.4	84	4.48	6300	87700	Ret	518
									R	1040
									W	10000
		Reconv.	21	36.7	80	4.42	5800	78300	Ret	820
									R	1406
									W	10000
		Reconv.	28	36.3	86	4.72	6800	94500	Ret	860
									R	1866
									W	10000
H. K. 33 years old ♀	Typhoid	Acme	31	39.1	79	4.15	7000	106400	Ret	402
									R	982
									W	10000
		Fever-abating	63	38.3	77	4.22	4800	136500	Ret	1396
									R	2938
									W	10000
		Reconv.	83	36.6	84	4.40	3300	157500	Ret	662
									R	1788
									W	10000
S. T. 22 years old ♀	Typhoid	Acme	28	39.1	62	3.16	2600	78400	Ret	1080
									R	3020
									W	10000
		Fever-abating	52	36.3	61	2.98	2500	69800	Ret	78
									R	3086
									W	10000
		Acme	60	39.4	54	2.64	3200	87450	Ret	210
									R	2390
									W	10000

of the Mitosis in Leucopenia

M. counts	M.I. %	Appearance of mitosis															
		Erythropoiesis							Leucopoiesis								
		Baso.	Polychr.	Oxy.	Pro.	Meta.	Ana.	Telo.	Mbl.	Unm. M.	Half-m. M.	Mat. M.	Meta-M.	Eos.	Pro.	Meta.	Ana.	Telo.
1	1.5																
26	48.5	4	8	14	16	4	2	4	0	2	8	8	0	0	4	8	4	2
18	1.8																
0	0																
38	35.5	4	14	20	12	4	4	18	0	2	6	4	0	4	6	2	4	4
17	1.7																
0	0																
76	40.0	16	32	24	32	16	8	20	0	4	10	8	0	0	14	6	2	0
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74	37.9	28	24	22	20	22	10	22	2	4	8	6	0	0	6	8	4	2
20	20.0																
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98	50.5	30	48	20	58	16	6	18	4	4	14	16	0	0	14	16	4	4
38	3.8																
0	0																
124	45.4	22	72	30	56	20	24	24	0	2	12	6	0	0	12	4	4	0
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72	38.6	20	28	24	38	16	8	10	0	9	10	6	0	0	18	4	0	2
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128	42.4	39	61	28	56	24	13	35	1	7	5	7	0	0	8	8	3	1
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86	35.9	14	28	44	34	18	14	20	2	4	6	4	0	0	2	6	6	2
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as an expression of sufficiency and decrease as that of insufficiency of the bone marrow.

Fig. 2 shows the relation between the irritability of the bone marrow to stimulus of the pathogenetic microbes and the reaction of the bone marrow. The curve of irritability of the bone marrow runs parallel to the curve of fever with some lag, and the curve of mitosis is illustrated as a rising curve in the first phase and a lowering curve in the second phase, as shown in Fig. 2.

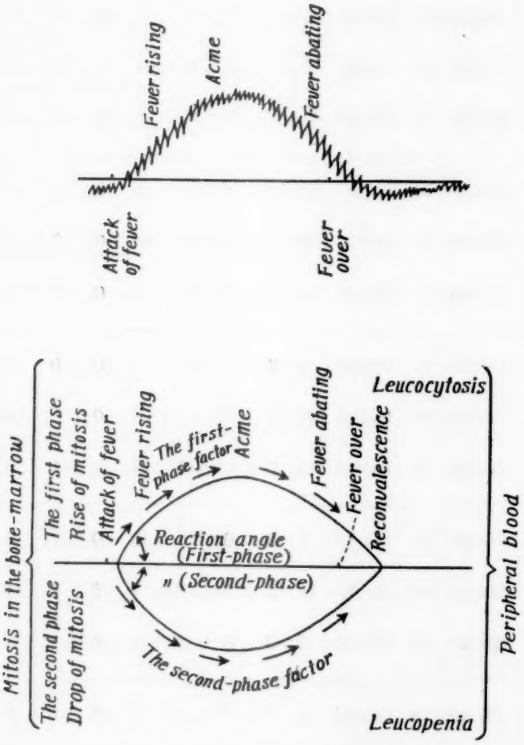

Fig. 2. Schemas of the irritability to stimulus and of the reaction mechanism of the leucopoietic system of the bone marrow.

If the angle that is made by a normal trend of mitosis and a trend toward increase or decrease of mitosis be called the reaction angle, the reaction angles in the first phase and the second phase are to be respectively called the first-phase reaction angle and the second-phase reaction angle; in the same way, a leucopoiesis in the first phase can be called a first-phase reaction and the one in the

second phase a second-phase reaction.

The reaction angle is proportionate to the specificity, quality and quantity of the pathogenetic factors and the disposition of individuals. The pathogenetic microbes causing leucocytosis belong to the first-phase factors and those causing leucopenia to the second-phase factors.

In the cases more than two factors are added up, if these factors belong to the same phase, the curve of mitosis elevates or drops more strikingly according to the summation of factors in the same phase, while if thesef actors belong to the different phases, the curve of mitosis changes its trend upwards or downwards according to the differences of factors, as shown in Fig. 3. For instance, in a typhoid fever case complicated with pneumonia or intestinal perforation, if the first-phase factor is greater than the second-phase factor, the trend of mitosis changes from a lowering to a rising tendency and the curve of mitosis changes accordingly as shown in Figs. 3–1, showing an increase of leucocytes both in the bone marrow and peripheral blood acco ding to the difference of the first- and second-phase factors. Figs. 3–2 show the opposite-tending curve in a scarlet

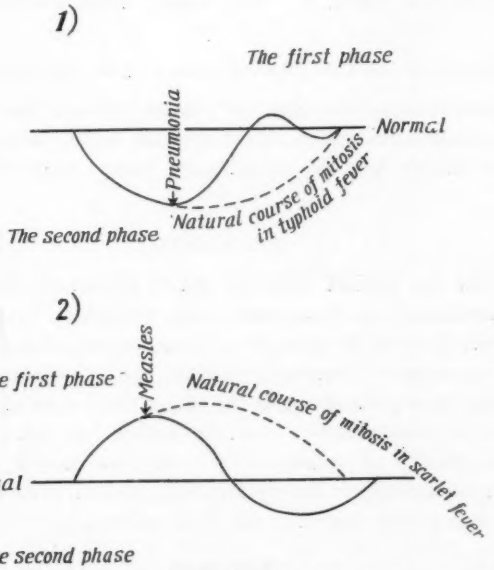

Fig. 3. 1). Case in the second phase, in which in the course of the illness a second-phase factor is complicated with a first-phase factor (typhoid fever case complicated with pneumonia).

2). Case in the first phase, in which vice versa in the course of the illness a first-phase factor is complicated with a second-phase factor (scarlet fever case complicated with measles).

fever complicated with measles.

C. Specificity of the two phase-factors

Here the specificity of stimulant factors is of great importance, and all stimulant factors of pathogenetic microbes and physico-chemical agents have their respective specificity not only biologically but also clinically. For example, the pathogenetic microbes of typhoid, paratyphoid A, paratyphoid K (salmonella Sendai) and paratyphoid B are called the germs of the typhoid group in our country and belong to the second-phase factors, but it can be found that even the factors belonging to the same phase factors they have all orderly and definitely the respective specificity, and that the reaction of the pathogenetic microbes against human body is the most active in typhoid bacilli, followed in descending order by paratyphoid A, K and B bacilli. The reaction angles of these second-phase factors are in the order of typhoid >paratyphoid A> paratyphoid K> paratyphoid B bacilli.

From these results it can be easily understood that in the peripheral blood leucopenia is the most striking in typhoid fever, moderate in paratyphoid A and K and slight in paratyphoid B. Also similar differences can be observed in leucocytosis.

D. Relation between the Two Phase Factors and the Autonomic Nerve System

In my observations concerning the relation between the two phase factors and the autonomic nerve system, the first-phase factor causes sympathicotonia with a rise of mitosis and the second-phase factor vagotonia with a drop of mitosis.

CONCLUSION

The author has studied with the aid of Miyamoto's laborious work the causative mechanism of leucocytosis and leucopenia in various infectious diseases in order to seek the principle of hematopoiesis of the bone marrow.

These observations of hematopoiesis of the bone marrow in the courses of infectious diseases have gradually made the author think of an idea of "PHASES" of mitosis in the bone marrow. And the author has come to the conclusion regarding the principle of hematopoiesis in the bone marrow, that erythrocytes are formed monophasically in the erythropoietic system, while leucocytes diphasically in the leucopoietic system in the bone marrow.

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On the Essential Nature of Hematopoietic Function of Bone Marrow

Report 2: On the Causative Mechanism of Leucocytosis and Leucopenia

By

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(Received for publication, March 14, 1961)

PREFASE

The principle of hematopoiesis in the bone marrow lies in the monophasic mitosis in the erythropoietic system and in the diphasic mitosis in the leucopoietic system, as discussed in my previous reports[1-6].

Excluding pathogenetic factors in infectious diseases, there are many physico-chemical factors which cause leucocytosis and leucopenia. These factors are as follows:

1. Leucocytosis

a) Chemical agents. Nucleic acid and other kinds of acid, potasium chlorate, phenylhydrazine, chrome: antipyretics, i.e. acetoanilide, phenacetine, camphor, digitalis,

b) Metabolic products. In uremia, diabetic acidosis, eclampsia, gout, burns and so on.

2. Leucopenia

a) Chemical agents. Benzol and its derivatives, e.g., aniline, amidopyrine, dinitrophenol, sulfonamide; gold, lead, mercury, illuminating gas, arsenic, salvarsan, thiouracil; anticancerous drugs: ruethane, nitrogen mastard, nitromin, 8-azaguanine, 6-mercaptopurine, carzinophyllin, mitomycine, azaserine and so forth.

b) Physical agents. Radium, thorium X, Röntgen-rays, ionising radation, etc.

It is well known that the factors which cause leucocytosis stimulate mitosis, while those which cause leucopenia inhibit it and that, when excessively put to use (intoxication dosis), both the factors become the cause of insufficiency of

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the bone marrow. Recently, Röntgen-rays, radium and isotopes are widely used in the purpose of diagnosis and therapy, and also anticancerous drugs have chemotherapeutic efficacy against malignant neoplasms in practical medicine. On the other hand, as a side-effect, leucopenia caused by dropping of mitosis in the bone marrow has come to be paid attention to lately.

The factors that cause leucocytosis belong to the first-phase factors and those that cause leucopenia to the second-phase factors.

As stimulant factors, these physico-chemical first- and second-phase factors do not differ from microbial first- and second-phase factors.

The hematopoietic function in the bone marrow is an expression of an adaptation of the internal environment (blood and lymph) attained in the course of the struggle of human body against the pathogenetic microbes.

Therefore, the principle of hematopoiesis in the bone marrow can be found typically illustrated in the course of infectious diseases.

Genetic Mechanism of Leucocytosis and Leucopenia

1. The hematopoietic function of the bone marrow

The hematopoietic functions of the bone marrow are divided into two kinds; the formation of the cells and their maturation.

a. Formation of the blood cells

In the case of leucocytosis, when the physico-chemical or pathogenetic first-phase factor has acted upon human body, the nearly matured cells which are on the right in the bone marrow and ready for flowing, flow out of the bone marrow into the blood in order to comply with the peripheral demand, and consequently a symptomatic leucocytosis with a nuclear shift to the left appears in the blood, at the same time a decrease of matured cells with a nuclear shift to the left being seen in the bone marrow.

In the meanwhile the bone marrow becomes excited by the stimuli of first-phase factors and the stimulation is transmitted from left to right in the bone

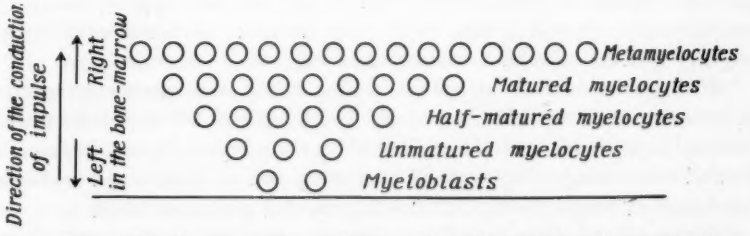

Fig. 1. The direction of the conduction of impulse to raise or lower mitosis of the nucleated cells in the bone marrow.

marrow, as shown in Fig. 1. That is, the mitosis of cells on the left in the bone marrow (primitive cells) rises causing increase of the cells, showing a nuclear shift to the left. After that a rise of mitosis accompanying an increase of cells shifts gradually from left to right in the bone marrow, as shown in Fig. 2.

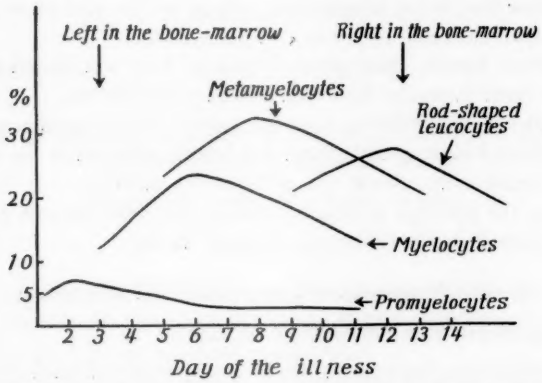

Fig. 2. The trend toward increasing the neutrophils in the bone marrow.

As observed above, in the bone marrow of leucocytosis the nucleated cells increase by a rise of mitosis. Especially, in the acme stage in infectious diseases, mitosis index showed the highest value of 3.28‰ and the center of mitosis reached the utmost right limit. Consequently, the matured cells on the right in the bone marrow made such a remarkable increase that they oversupplied the demand and destruction in the periphery and caused leucocytosis in the periphery, and yet preserving a number of reserved cells in the bone marrow to meet further demand of the periphery, so long as the bone marrow was sufficiency. In the fever-abating stage, mitosis of the leucopoietic system in the bone marrow lowered by degrees and the leucocytes diminished in quantity; mitosis index took the subnormal value of 2.30‰, as observed in the previous report[1], but in the reconvalescence showed 2.58‰, while the demand and destruction in the periphery gradually abated to the normal level.

After reconvalescence, as shown in Fig. 3. the bone marrow turned from the irritation state to inhibition state, and a nuclear left-shift took place in the bone marrow and a nuclear right-shift in the peripheral blood, forming the so-called mirror image. Appearances of over-ripened neutrophils and eosinophils were observed frequently in the bone marrow and peripheral blood.

In the case of leucopenia, when the physico-chemical or pathogenetic second-phase factor has acted upon the human body, the reserved leucocytes flow out of

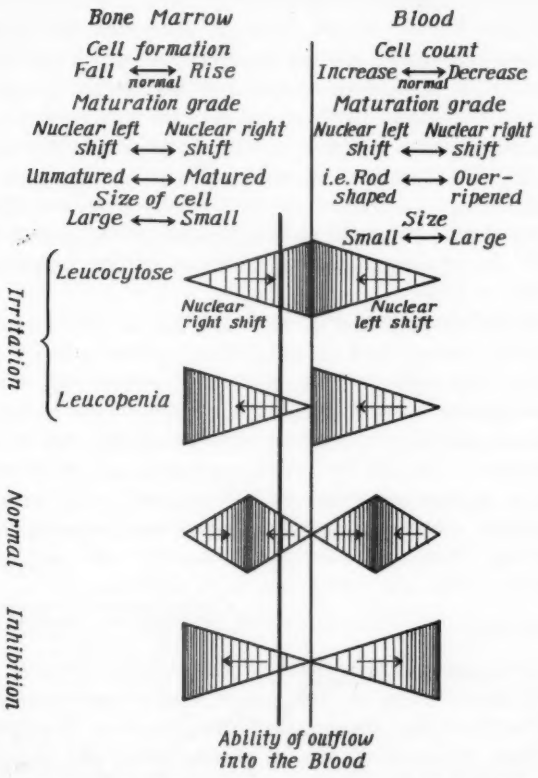

Fig. 3. Schema of cell formation and mechanism of its regulation.

the bone marrow into peripheral blood, showing a decrease of the nucleated cells with a symptomatic nuclear left-shift in the bone marrow and a symptomatic leucocytosis with a nuclear left-shift in the peripheral blood, as those seen in the case of leucocytosis.

In the meanwhile by the stimuli of the second-phase factors, droping of mitosis accompanying a diminition of the nucleated cells occurs at first in unmatured cells in the bone marrow. The drop of mitosis with a decrease of the cells shifts gradually from the left to the right in the bone marrow. The mitosis index lowers to 1.94‰ in the acme stage of the infectious diseases and, in the bone marrow, the nucleated cells remarkably diminish in quantity both by the mobilization of the cells into the peripheral blood and by the dropping of mitosis; especially, segmented and rod-shaped neutrophil leucocytes in the bone

marrow abate most remarkably in the latter half of acme and fever-abating stage. Thereupon Barta[7] termed the bone marrow in the fever-rising stage "a metamyelocytic marrow", that in the peak stage and fever-abating stage "a myelocytic, rarely myeloblastic marrow", Henning[8] called that "a strongly left shifted one" and Storti and Filippi[9] named it "a cell-poor marrow" and nucleated cells in the bone marrow diminish in high degree and relative lymphocytosis appeared in the bone marrow in the acme and fever-abating stage.

The same changes were also observed in the peripheral blood: obvious leucopenia was seen in the acme and fever-abating stage, often decreasing to 1.800–2.000 in leucocyte count, accompanying a relative lymphocytosis which sometimes rose to 80–90%.

In the fever-abating stage, M.I. showed 2.52‰, a little higher than normal value, and in the reconvalescence 2.26‰, almost normal value.

Thus, in leucopenia the dropping of mitosis is conspicuous in the acme, and after the fever-abating stage mitosis gradually rose from left to right in the bone marrow and returned to normal. What is worthy of attention in the peripheral blood in leucopenia is that this mechanism causes that even in the reconvalescence in which the leucocyte count recovered to the normal value, neutrophils especially its segmented neutrophils showed a conspicuous decrease, a nuclear left-shift took a long while, and overripened leucocytes did not appear. Fig 3. indicates cell formation and mechanism of its regulation.

b. Maturation

Although the genetic mechanism of leucopenia has been attributed to the disturbance of maturation in the bone marrow with the appearances of basophilic stipplings of erythrocytes, anisocytosis, toxic granules of leucocytes and Döhle's bodies, yet these appearances, are, as already observed, noticeable both in leucocytosis and leucopenia and not peculiar to leucopenia alone.

2. Out-flow

The genetic cause of leucopenia is considered to be the disturbance of out-flow of leucocytes from the bone marrow into the peripheral blood. This out-flow function is related not to the regulation of the bone marrow but, as Rohr[10] mentioned, to the potential falling gradient between the bone marrow and peripheral blood: the one is in the bone marrow and the falling gradient becomes steep by an acceleration of the formation, while the other is in the peripheral blood and the falling gradient becomes steep by an increase of consumption and demand of the periphery.

The slope of this falling gradient determines the strength of out-flow function, and also the height of out-flow speed of the blood cells from the bone marrow into peripheral blood. The larger is the falling gradient, the more

conspicuous becomes a leucocytosis in the peripheral blood, and the more strongly appears a nuclear left shift.

That is to say, both in leucocytosis and leucopenia, the more acute and severe an attack of illness, the larger the consumption and demand of the periphery, and accordingly the steeper the falling gradient. Consequently, many younger cells flow out of the bone marrow into peripheral blood and at the early stage of the illness symptomatic leucocytosis with a nuclear shift to the left becomes conspicuous both in leucocytosis and leucopenia, and accordingly, a decrease of the matured cells on the right in the bone marrow and a symptomatic nuclear shift to the left in the bone marrow become obvious (Figs. 4 and 5).

The appearance of these younger cells in the peripheral blood is not necessarily related to the quantity of nucleated cells in the bone marrow. In leucocytosis, they appeared in the peripheral blood only in the course when nucleated cells in the bone marrow were increasing, as shown in Fig. 6. Also in leucopenia, as shown in Fig. 7, they appeared in the peripheral blood only when peripheral leucocytes were in the decreasing course.

The potential falling gradient between the bone marrow and peripheral blood became less steep after the fever-abating stage.

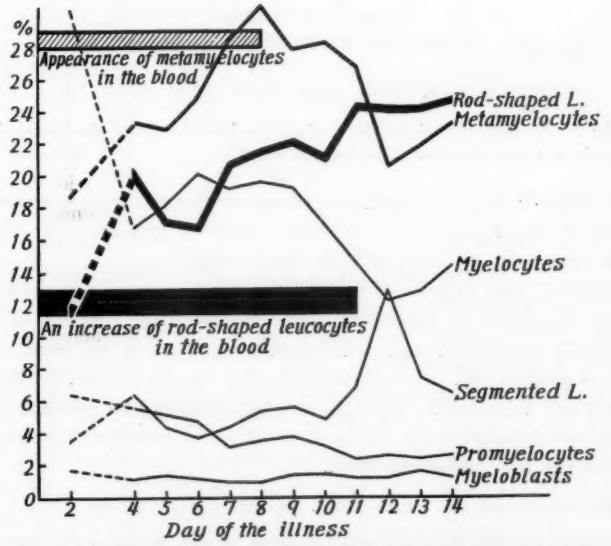

Fig. 4. Change of various kinds of the nucleated cells of the leucopoietic system in the bone marrow of leucocytosis cases.

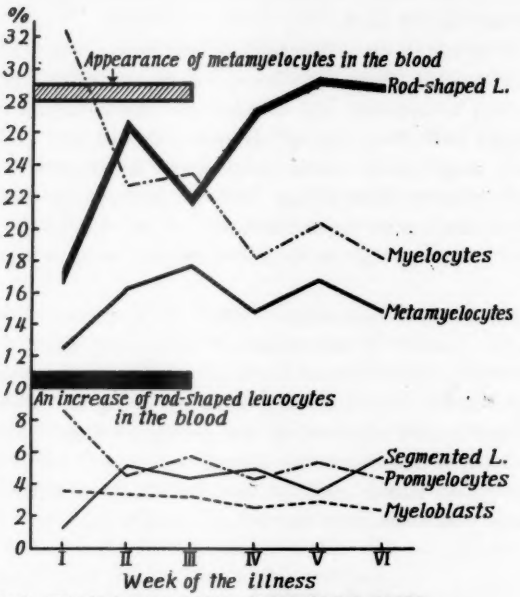

Fig. 5. Change of various kinds of the nucleated cells of the leucopoietic system in the bone marrow of leucopenia cases.

In leucocytosis the falling gradient became smaller in the bone marrow and in the peripheral blood on level of a large count of granulocytes, while it in leucopenia became so on level of their small count.

Galinowski[11] and Fujimori[12] thought the genetic cause of leucopenia in the disturbance of out-flow of the blood cells. However, the decrease of nucleated cells, especially segmented and rod-shaped leucocytes, in the bone marrow in leucopenia is greater, and therefore the cells that can be applied for the demand of periphery are diminished. From these facts it cannot be recognized that the genetic cause of leucopenia lies in the disturbance of out-flow of leucocytes from the bone marrow into peripheral blood.

3. The destruction of the white blood cells in the periphery

There is an opinion that the genetic cause of leucopenia is the destruction of white blood cells in the periphery. Faludi[13] explained the genetic cause of leucopenia by suppuration within the blood vessels. That is, the decrease of

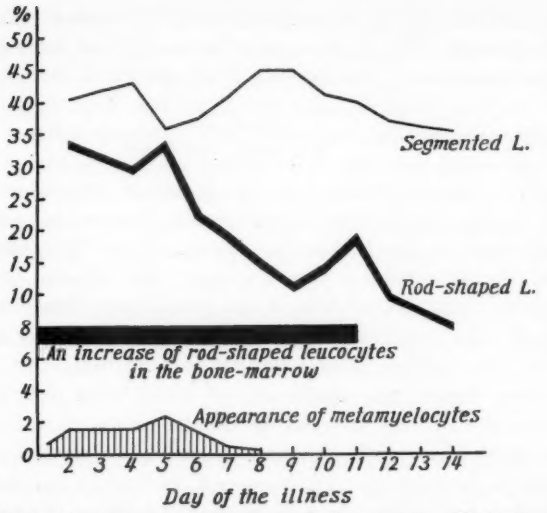

Fig. 6. Appearance and increase of the younger cells in the peripheral blood of leucocytosis cases.

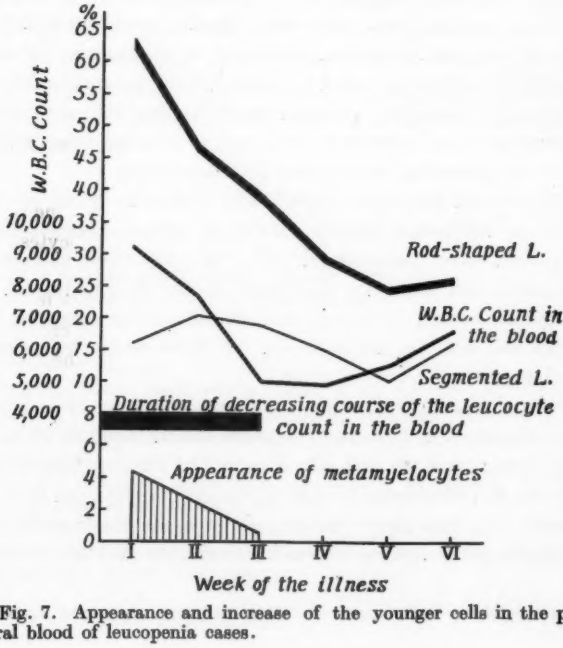

Fig. 7. Appearance and increase of the younger cells in the peripheral blood of leucopenia cases.

leucocytes in number is caused by a destruction of the white blood cells in the peripheral blood vessels. Should this opinion be true, mitosis index in leucopenia must be higher than that in normal cases. Accordingly, in the formula

$$\text{Mitosis index (‰)} = \frac{\text{Mitosis Count}}{\text{Nucleated Cell Count}} \times 1{,}000$$

the mitosis index must be greater than that in normal person, so long as the mitosis count is not smaller than normal count, because in leucopenia the nucleated cell count of the leucopoietic system in the bone marrow is fairy decreased and the denominator is naturally small. Nevertheless, in the acme in leucopenia mitosis index shows 1.94‰, which is lower than normal value (2.40‰). According to the above-mentioned formula this can be true only by a lowering of mitosis. So, the Faludi's opinion can not be admitted.

In infectious disease, the destruction of white blood cells is accelerated according to the severity and progress of the disease, so that the destruction is higher in severe cases with prolonged course. Yet, no difference can be observed between leucocytosis and leucopenia so long as insufficiency of the bone marrow does not secondarily take place. The degree of destruction and degeneration of nuclei and protoplasma, such as vacuole formation or appearance of toxic granules, is not regarded to be particularly higher in leucopenia.

In the bone marrow and peripheral blood, lymphocytes decreased relatively in leucocytosis and increased relatively in leucopenia. Enlargement of spleen is tangible in leucopenia caused by typhoid fever, paratyphoid fever A. K. B, Japanese river fever, Kala-azar, etc., and is also noticeable in leucocytosis caused by sepsis, relapsing fever, eruptive typhus, etc., so that the enlargement of spleen does not serve in discerning leucocytosis and leucopenia.

As Fraenkel[14] and Hartwig[15] autoptically observed, organic alterations such as necrotic foci or destruction pictures are seen in the bone marrow in leucopenia caused by typhoid fever, paratyphoid fever, etc., but these necrotic foci are a part of the necrotic foci formed in every part of the whole body, and can be seen also in leucocytosis caused by sepsis and the like. Consequently, leucocytosis and leucopenia can not be distinguished by the existence of necrotic foci or destruction pictures in the bone marrow.

Physico-chemical agents constitute the next question. When these factors operate upon the bone marrow, there appears the monophasis of mitosis in the erythropoietic system and the diphasis of mitosis in the leucopoietic system, and both leucocytosis and leucopenia seem to be the results projected in the peripheral blood. Namely, the first-phase factors raise mitosis showing the first-phase reaction, while the second-phase factors lower mitosis showing the second-phase reaction.

Specificity of these factors can be found not only between these two phase

factors but also between the factors of the same phase, and the differences are noticeable between leucocytosis and leucopenia in their degrees. For example, it is well-known that, among the second-phase factors themselves, the agents such as Röntgen-rays, thorium X, radium, nitrogen mustard, nitromin, 8-azaguanine, urethane, carzinophyllin, benzol, gold and others never fail to lower the mitosis in the leucopoietic system, and that they make mitosis abnormal when excessively used; while the agents such as amidopyrine, sulfonamide, antispasmodic drugs, antimicrobial drugs, antithyroid drugs often cause severe leucopenia according to the person's constitution, though usually it is only mild and sometimes does not appear at all.

Thus, the difference in the degree of leucocytosis and leucopenia appears in accordance with the specificity of the stimulant factors, their qualitative and quantitative difference, and also with the individual difference.

CONCLUSION

The essential difference between leucocytosis and leucopenia consists in the diphasis of mitosis in the leucopoietic system in the bone marrow and not differences in their maturation in the bone marrow, in the out-flow of white blood cells out of the bone marrow into the peripheral blood, and in the destruction of leucocytes in the periphery. The essential difference between the two originates in the difference of mitosis of the leucopoietic system in the bone marrow; in leucocytosis, the mitosis rises and the leucocytes consequently increase, while in leucopenia, the mitosis lowers and the leucocytes consequently decrease. Leucocytosis and leucopenia occur by a projection of these mechanism into the peripheral blood.

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On the Essential Nature of Hematopoietic Function of Bone Marrow

Report 3 : On the Field of the Blood Defense Reaction

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(Received for publication, March 14, 1961)

PREFACE

The author has found that the principle of hematopoiesis in the bone marrow consists in the monophasic formation in the erythropoietic system, and in the diphasic formation in the leucopoietic system. Here the blood reactions must be duly apprehended in order to apprehend this principle duly.

From Pithecanthropus downward, the human body has been evolving and changing in an incessant struggle against microbes, during the fifty myriad years of the course of evolution. leucopoietic reaction in leucocytosis and leucopenia is one of a living creature's reactions obtained by an adaptation to the environment. Therefore, the observation of the blood reaction in the infectious diseases is the most rational way to study the principle of hematopoiesis in the bone marrow.

Reaction Fields of Blood Cells and Serum Observed from the Angle of Internal Environment

In infectious diseases, leucocytosis can be easily understood from the function of the white blood cells in the defense mechanism of the body, but leucopenia is seemingly inconsistent with this notion, for the decrease of the white blood cells means the decline of defense force against pathogenetic microbes.

This problem must be inquired from the point of bacterio-immunology, apart from the point of hematology.

In infectious diseases, there are differences between coccal infection and bacillary infection ; increase of the white blood cells is larger in coccal infection than in bacillary infection, while on the contrary the production of antibodies in the serum is higher in the latter than in the former. In general, production of antibodies is active in the diseases involving leucopenia. Now the blood reactions

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partaking in the defense mechanism of the body is divided into the leucocyte-bacterium reaction and the serum-bacterium (antibody-antigen) reaction.

The followings are the experimental results obtained in the entire course of typhoid fever. In 536 typhoid fever cases during the term from entering to leaving hospital, the author cultivated typhoid bacilli 1727 times sampled from the bone marrow, blood, stool, urine and pharyngeal mucus at the rate of once a week regularly. Picking out 3 cc of bone marrow specimen by bone-marrow puncture, putting it into 5–7 cc of bile media, and after enrichment for 24 hours therein it was painted on the surface of the Endo's media and cultivated at 37°C. This procedure was repeated every day until the pathogenetic microbes were isolated as colonies, and when the cultures turned out to be negative after 5 day's enrichment in bile media I determined them to be negative. And at the same time, Widal's reaction of serum and peripheral blood was also examined. Fig. 1. presents the relation between the development of the bacilli in the bone marrow and the titers of Widal's reaction, obtained by these systematic serial examinations. The percentage of positive finding of the pathogenetic microbes is much higher in the bone marrow than in the blood, and is the highest at the early stage of the illness; in the first week of the illness, the percentage of positive findings was 91.9% in the bone marrow and 76.6% in the blood. Then the percentage gradually abated during its courses, but the curve of positive findings of the bacilli in the bone marrow dropped more slowly than in the blood. On the contrary, the Widal reaction presented nothing characteristic at the onset of the illness, but become positive during the second week and reached its

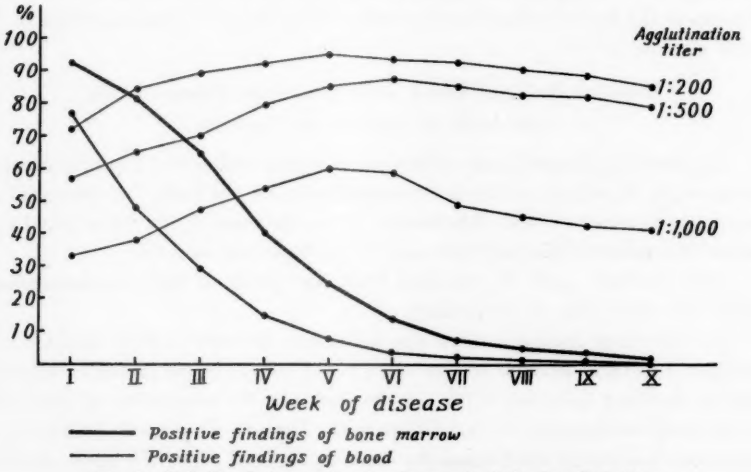


Fig. 1. Relation between the development of the bacilli in bone marrow and Widal reaction in 536 typhoid fever cases.

maximal height at about the 4th to 6th week. The percentage of positive findings of pathogenetic microbes in the bone marrow and in the blood was in inverse relation to the titers of agglutination of sera.

Fig. 2. shows the relation between the leucocyte count and the titer of agglutination of serum; they are in inverse relation to each other, operating as mutual antagonists.

The defense force of the blood is divided into the defense force of leucocytes and the defense force of serum, which are in inverse relation to each other. The fields in which these defense forces operate, the reaction fields, are divided into the field of leucocyte-bacterium reaction in which leucocytes take part, and the field of serum-bacterium reaction in which serum takes part (Fig. 3.). In a broader sense, the former is the field of the cell-bacterium reaction and the latter is the field of antibody-antigen reaction. The selection of the main field of struggle between pathogenetic microbes (parasites) and living body (host) is determined by the specificity of the pathogenetic microbes. The strength of reaction is represented by the width of reaction as shown in Fig. 3. From the point of strength of reaction, these reaction fields are divided into I) the region in which leucocyte-bacterium reaction is dominant, II) the region in which leucocyte-bacterium reaction and serum-bacterium reaction are equal and III) the region in which serum-bacterium reaction is dominant.

When the reaction fields are illustrated as divided into two oval fields, namely the field of cell-bacterium reaction and the field of antibody-antigen reaction, they form the three regions, one overlapping the other, as shown in Fig. 3.

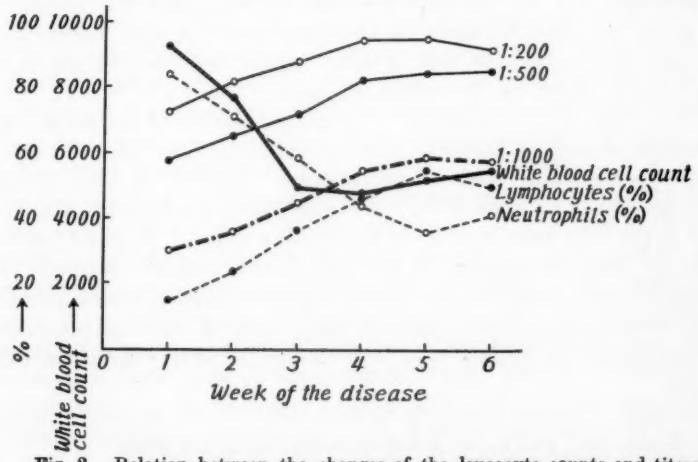
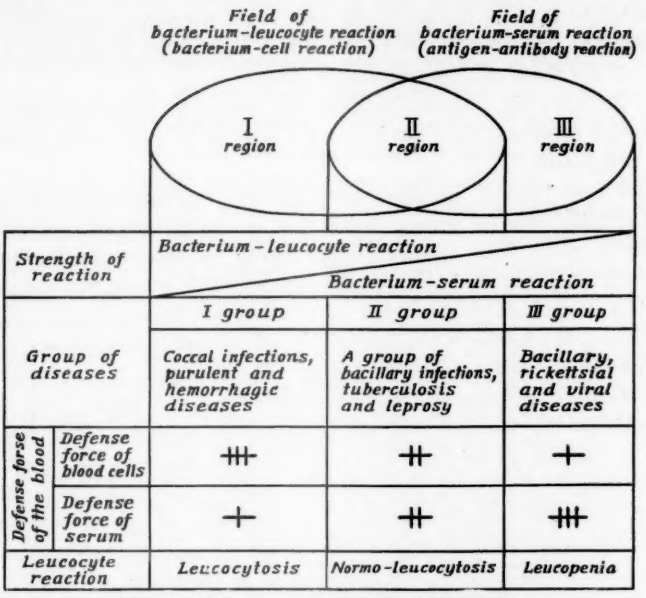


Fig. 2. Relation between the changes of the leucocyte counts and titers of agglutination of the peripheral blood, obtained through 1727 examinations in the courses of 536 typhoid cases.



Strength of reaction	Bacterium-leucocyte reaction / Bacterium-serum reaction		
	I group	II group	III group
Group of diseases	Coccal infections, purulent and hemorrhagic diseases	A group of bacillary infections, tuberculosis and leprosy	Bacillary, rickettsial and viral diseases
Defense force of the blood: Defense force of blood cells	+++	++	+
Defense force of the blood: Defense force of serum	+	++	+++
Leucocyte reaction	Leucocytosis	Normo-leucocytosis	Leucopenia

Fig. 3. Defense reaction fields of the blood.

That is to say, in the I region the serum-bacterium reaction does not appear in the foreground and the leucocyte-bacterium reaction plays the leading part while in the III region the leucocyte-bacterium reaction is weak and the serum-bacterium reaction plays the lead; in the II region both reactions are in equal strength and is shown by the overlapped region.

The next point to be observed is the defense reaction of the blood. When the struggle between pathogenetic microbes and living body occurs in the field of leucocyte-bacterium reaction (cell-bacterium reaction) by the first-phase factors, demand of the white blood cells is accelerated, mitosis in the leucopoietic system in the bone marrow rises, the first-phase reaction is presented, nucleated cells in the leucopoietic system in the bone marrow increase. Similarly, when the said struggle begins in the field of serum-bacterium reaction (antibody-antigen reaction) by the second-phase factors, demand of antibodies is accelerated and consequently the production of antibodies in the whole body is remarkably increased.

The defense-reaction energy is applied chiefly to the leucocyte formation in the former case, and to the antibody formation in the latter.

When the above-mentioned reactions are viewed from the side of peripheral leucocytes, they mean that leucocytosis occurs in the I region and leucopenia

appears in the III region. In the II region the strength of the cell-bacterium reaction and the antibody-antigen reaction being almost equal, the leucocyte count shows an almost normal value (normoleucocytosis). Herein, therefore, lies the reason that in infectious diseases caused by tuberculous or leprous bacilli the leucocyte count takes an almost normal value.

However, the reaction does not take place in the II region from the very onset of tuberculous infectious diseases. The defense reaction of the blood against the tuberculous bacilli takes place in the I region at the early stage of illness when the production of antibodies is nil or only very limited, and leucocytosis is induced in the peripheral blood. But abreast with the conspicuous increase of the production of antibodies and gradual recovery of the illness, the leucocyte count shows as almost normal value with neutropenia and the deficit of the reduction in the number of neutrophils below normal is made up by an increase in lymphocytes, the reaction shifting to the II region. In exudative tuberculosis and miliary tuberculosis, the reaction takes place in the I region, and furthermore the reaction field shifts from the II to the I region abreast with aggravation of the illness, and shifts again from the I to the II region by the improvement of it.

Thus, the field of the leucocyte reaction in the blood in struggle of the body against the tuberculous bacilli shifts repeatedly between the I and II region, under the mutual relation of the power of pathogenetic microbes and of the resisting force of the body.

Furthermore, in severe cases such as miliary tuberculosis the leucocytes remarkably decreased in number without improving, these cases simply signify the true insufficiency of the bone marrow and the destruction of the defensive force by an overwhelming attack of pathogenetic microbes. In general, by the previously mentioned mechanism, in tuberculous infection the leucocyte count showed normal value under the condition of neutropenia and lymphocytosis in the case the disease took favourable course, and leucocytosis appeared in the case it recurred or aggravated.

In typhoid fever in which leucopenia appears most obviously, the defense reaction does not take place in the III region from the very onset of the illness. It occurs in the field of leucocyte-bacterium reaction at the onset of the illness when antibodies are not yet produced, so that the demand of the periphery accelerates, the potential falling gradient becomes steep, reserved neutrophils in the bone marrow flows out into the peripheral blood, and as the results there appears a symptomatic leucocytosis in the peripheral blood. Namely, the reaction field is in the I region as shown in Fig. 3. Thereafter, the production of antibodies is gradually increased and the reaction field shifts to the II region at the period from the end of the first week to the beginning of the second week of the disease, and the leucocyte count decreases to the range of normal value. At the period from the end of the second week to the beginning of the third week of the disease,

the antibodies are actively produced, the leucocyte count is reduced more and more, and the reaction field shifts into the III region. At the period from the end of the third week to the fourth week of the disease, the leucocyte count shows the lowest value. In short, the leucocyte count and the titer of agglutination are antagonistic to each other throughout the entire course of the disease, and the leucocyte count takes the lowest value in the third — fourth week of the disease and the titer of agglutination (antibodies) takes the highest value in the third — sixth week of the disease, as presented in Fig. 2.

Thus, the defense force of the leucocytes and serum are in inverse relation, operating as mutual antagonists.

Here the manner and the function of lymphocytes are worthy of attention. In the peripheral blood in normal person, lymphocytes show 38% (25–45%), the percentage of which is the highest next to 55% of neutrophils, and the sum 93% forms the largest majority of the leucocytes. In infectious diseases, lymphocytes are in inverse relation to neutrophils as shown in Fig. 2, and it decreases in number relatively when symptomatic leucocytosis appears at the onset of the disease, and increase in the course of the disease in parallel to the titer of antibodies in the serum.

Generally speaking, lymphocytes decrease relatively both in leucocytosis and in leucopenia in number at the onset of the disease and increase at the stages from the fever-abating to the reconvalescence, and the increase of lymphocytes is regarded as an indication of recovering phenomenon in infectious diseases. Accordingly, neutrophils plays the leading part in the field of leucocyte-bacterium reaction while lymphocytes play the leading part in the field of antibody-antigen reaction. Considering these facts, it is evident that lymphocytes are partaking in the antibody formation.

Thus a constant antagonistic relation is observable between neutrophils and lymphocytes, both of which form a large majority of leucocytes. In the field of the defense reaction of the blood, a defensive net is widely spread out extending over from the field of leucocyte-bacterium reaction to the field of antibody-antigen reaction, and each mesh is filled up with minor relations. The existence of the two reaction fields, the fields of leucocyte-bacterium reaction and the field of antibody-antigen reaction in the defense reaction of the blood, is related with the diphasis of mitosis in the leucopoietic system in the bone marrow.

The relation is shown in Table I. The phases of mitosis come forth to fill the object of the fields of defense reaction, so that the phases are co-operative with the fields. Accordingly the defense reaction of the blood is an adaptation to the internal environment in the body, and the leucocytosis and leucopenia are the symptoms when the appearances of adaptation are veiwed from the point of the peripheral blood.

Here the relation between the phases and the fields requires further attention.

TABLE I. Relation between the Hematopoietic Phases in the Bone Marrow and the Reaction Fields of the Blood

Hematopoietic system in the bone marrow	Phases of mitosis in the bone marrow		Reaction fields of the blood	Changes of blood cells in the peripheral blood
Erythropoietic system	Monophasis of mitosis		O_2 -Metabolism..one field	Trend toward normal value
Leucopoietic system (Neutrophil series)	Diphasis	1st phase (Rise of mitosis)	Blood cell-bacterium reaction (Cell-bacterium reaction) } 2 fields	Leucocytosis
		2nd phase (Drop of mitosis)	Blood serum-bacterium reaction (Antibody-antigen reaction)	Leucopenia

Viewed from the point of phases, the trend of mitosis in the leucopoietic system in the bone marrow turned from the second phase to the first phase in the case when typhoid fever was complicated with pneumonia, and the trend of mitosis turned from the first phase to the second phase when scarlet fever was complicated with measles; while viewed from the point of fields, the main reaciton field shifted from the field of antibody-antigen reaction to the field of leucocyte-bacterium reaction in the former, and in the latter from the field of leucocyte-bacterium reaction to that of antibody-antigen reaction.

As for erythrocytes, however, there is only one reaction field for them, because the function of erythrocytes is for gas metabolism of absorbing 0^2 and releasing CO^2, and because the metabolism of gases is not performed by anyone else. Therefore, when anemia occurs without accompanying insufficiency of the bone marrow, the metabolism of gases is not fully performed and the CO^2 concentration in the whole body is raised, which stimulates the erythropoietic system in the bone marrow causing the rise of mitosis. That is, a rise of CO^2 concentration reacts to the erythropoietic system as a stimulant factor; and by such means, erythrocyte count increases to maintain its normal value. Thus the reaction field of the erythrocytes being only one, the mitosis of the erythropoietic system in the bone marrow takes one phase and the erythropoietic system is always in an effort to support the normal value of erythrocyte count, unless the bone marrow is in insufficiency.

As I have observed above, the erythrocyte reaction takes one field while the leucocyte reaction takes two fields, and in order to fill up the object of the fields, erythrocytes take one phase while leucocytes take two phases in the formation of the blood cells in the bone marrow.

It means a favourable course of the disease with a good prognosis, unless too extreme, to see an increase of the leucocytes in a course of the disease, which

usually accompanies leucocytosis and their decrease in the course of a disease, usually accompanying leucopenia. On the contrary, it is not a favourable course of the disease, unless it is very mild, to find the leucocytes decrease in the course of the disease, which as usual shows leucocytosis, because it proves insufficiency of the bone marrow or complications with a serious prognosis and similarly not favourable, to find them increase in the course of the disease, which as usual shows leucopenia, because it argues complications.

Accordingly, the leucocyte formation in the leucopoietic system in the bone marrow is presented, as shown in Table II.

TABLE II. Hematopoiesis of the Leucopoietic System of the Bone Marrow in Infectious Diseases

Hematopoiesis of the bone marrow	1. Sufficiency	1. Rise of mitosis	The first phase	Leucocytosis
		2. Drop of mitosis	The second phase	Leucopenia
	2. Insufficiency	Functional / Organic		1. Extremely severe cases, which primarily belong to leucocytosis but show remarkable leucopenia; sepsis, pneumonia, perityphlitis.
				2. Extremely severe cases of leucopenia, in which the white blood cells do not increase not-withstanding severe complication by perforative peritonitis or pneumonia.

In the case when the symptoms are particularly severe owing to the quality and quantity of the stimulant factors and by the individual difference of the body, the insufficiency of the bone marrow appears both in leucocytosis and in leucopenia. In the leucocytosis the leucocyte count decreased and the prognosis was unfavourable, as in the cases of serious diseases such as sepsis, pneumonia, perityphlitis and tuberculosis. In leucopenia, though the prognosis was equally unfavourable in the case when, the leucocyte count was not increased, in spite of the severe complication such as pneumonia, intestinal perforation or other purulent diseases with severe typhoid, yet the phenomenon is secondary leucopenia caused by insufficiency of the bone marrow. This leucopenia is an expression of an incapability of adaptation of the body to the internal environment, therefore, and it is quite different in nature and should be strictly distinguished from primary leucopenia in biological significance.

As already mentioned, physico-chemical factors, besides infectious factors, also cause leucocytosis and leucopenia. In former days, the first-phase factor of sodium nucleinate was used at the examination of the bone marrow function to cause evident leucocytosis. Recently, the second-phase factors such as radium, thorium X, nirtogen mastards, nitromin, urethane, azaguanin, etc.

are lime-lighted as physico-chemical remedies for treatment of malignant neoplasms such as cancer, sarcoma and leukemia.

On the other hand, however, leucopenia as a side-effect of these means has come to be taken seriously. When used excess, these physico-chemical agents cause insufficiency of the bone marrow and the intoxication become obvious; but when moderate or proper doses of these agents are used, mitosis lowers correspondingly with the specificity and qualitative and quantitative difference of the applied factors, and with the individual difference of the body, leucopenia is produced in the peripheral blood. Accordingly, it is said of the second-phase factors such as anticancerous drugs used for therapeutic purpose, that the more the difference between the medical dose and intoxication dose is large, the more the utility value is great.

In the case of a patient in whom leucocyte count dropped remarkably by the usage of the anticancerous drugs, Röntgen-rays or radium, when pneumonia or purulent diseases come into complication and the patient becomes feverish thereupon, the leucocyte count turns toward increase. This case, similar to the case in which leucopenia of typhoid fever was complicated with pneumonia or other diseases, is a case in which the first-phase factors of pathogenetic agents were added to the second-phase factors of physico-chemical agents.

These phenomenon signifies that the main reaction field changes from the field of antibody-antigen reaction to the field of leucocyte-stimulant-factor reaction in accordance with the difference of the second- and the first-phase factors.

CONCLUSION

Regarding the reaction fields of the blood in the adaptation process of the body to the internal environment, erythrocytes have only one field of O^2 metabolism. The defense reaction field of the blood is divided into the field of leucocyte-stimulant factor reaction and the field of antibody-antigen reaction.

Leucocytes perform the most rational and suitable defense reaction under the constant antagonistic relation with serum in adaptation to the internal environment.

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On the Essential Nature of Hematopoietic Function of Bone Marrow

Report 4: On the Biological Significance of the Hematopoietic Phases of the Bone Marrow and the Fields of Blood Reaction from the Standpoint of Internal Environment

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PREFACE

Homosapiens has been evolving to a high degree in the course of repeated phylogenesis and ontogenesis for fifty myriad years from Pithecanthropus, and for 50–60 million years retrospectively since the age of mammalia. The defense force of the body against pathogenetic microbes is one of the forces of adaptation to the internal environment that were thus acquired in these evolutive courses. The defense forces of the body consist of both the defense force of the tissues (mass of cells), and the defense force of the humor; the former performs the cell-bacterium reaction and the latter the antibody-antigen reaction. As for the blood, the blood cells can be regarded as a free form of the tissues, and serum as a sort of humor. Accordingly, the defense force of the blood is one of the defense forces of the body, and is divided into the defense force of the white blood cells and the defense force of the serum. And in the course of the struggle against pathogenetic microbes, the two fields, namely the field of cell-bacterium reaction and the field of antibody-antigen reaction, were prepared as the chief battle-grounds of the two defense forces[1–18].

Biological Significance of Antibody-Antigen Reaction

There are leucocyte-bacterium reaction (cell-bacterium reaction) and serum-bacterium reaction (antibody-antigen reaction) in the defense reactions of the blood and, though the leucocyte-bacterium reaction is easily apprehended, the antibody-antigen reaction is apt to be neglected hematologically.

Even in the cases when not only microbial factors but also physico-chemical factors act as stimulant factors to the body, the defense reaction of the blood is still limited to the cell-stimulant factor reaction and antibody-antigen reaction. Therefore, if all the stimulant factors that attack from outside the body are in-

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cluded as stimulant factors in broader sense, then the bacteria are replaced by the stimulant factors and the reaction field can be shown with two fields; the field of leucocyte-stimulant factor reaction and the field of antibody-antigen reaction in broader sense.

The above-mentioned opinion about the limitation of the reaction field is due to the following reason that chemical factors appeared in the human society only two hundred odd years ago with a few exceptions, and that it is only some sixty years ago that physical factors such as the radiogen factors including X-rays were discovered; therefore, since these years are far shorter as compared with the fifty myriad years of human phylogenesis, since the age of mammalia, there is no believing of newly acquired ability of adaptation.

I have already described the antibody-antigen reaction of microbial factors in infectious diseases, on the principle of hematopoiesis in the bone marrow[1-8]. Therefore, the question here is about the antibody-antigen reaction that takes place when physico-chemical factors are taken for antigen, because the second-phase factors are indispensable for the purpose of diagnostic and therapeutic praxis.

Recently the field of utilization of radiogens such as Roentgen-rays and radium have been rapidly expanded year after year, and the appearance of isotopes has made a new epoch as tracers of metabolism, in the researches on metabolic function.

In additon, the effects of atom bomb and hydrogen bomb upon the hematopoietic function in the bone marrow have raised attention and fears of the world. On the other hand, chemical agents such as anticancerous drugs are now widely utilized either by themselves or together with physical agents in the clinical practice. Thus the physico-chemical agents have been proved their brilliant effectiveness, while on the other hand, their side-effects upon the hematopoietic function in the bone marrow and the biological significance must be examined anew.

As already described, the chief battle-field of the defense reaction is the field of leucocyte-stimulant factor reaction when the first-phase factors of physico-chemical agents have attacked the body, and on this occasion the antibody formation is so slight as to be of little importance.

On the contrary, when the second-phase factors of physico-chemical agents have acted on the body, the main field of the defense reaction is the field of antibody-antigen reaction. Accordingly the antibody production is presumed to be remarkable. It is well known that the usage of Roentgen-rays, radium, anticancerous drugs, etc. causes drop of mitosis of the leucopoietic system in the bone marrow and also causes leucopenia in the peripheral blood, and it is often also experienced that in the case of a patient in whom leucocyte count dropped remarkably by the usage of the anticancerous drugs, X-rays, radium, etc., when pneumonia, tonsillitis or other purulent diseases come into complication, and the

leucocyte count turnes toward increase. This case is similar with the case in which typhoid is complicated with pneumonia. That is to say, these cases do not mean the change of the bone-marrow function from insufficiency to sufficiency, but, mean that the bone marrow is irritated by and responds to the stimulation of the second-phase factors and that the mitosis in the bone marrow drops according to the quantitative and qualitative differences of the factors and to the individual differences, with the consequent reduction of leucocytes. This becomes clearer when we observe the increase in leucocyte count upon complication of the first-phase factors.

Of course, when these second-phase factors are used in great quantity the bone marrow is disturbed functionally and organically, and the leucocytes can no longer increase even by discontinuing their usage or by any complications. In this case, real insufficiency of the bone marrow comes to appear, quite as the secondary bone-marrow insufficiency in infectious diseases.

Thus the bone marrow makes quite the same response both to the physico-chemical factors and to the microbial factors, so that the two factors do not differ essentially as the stimulant factors to the bone marrow. In other words, it is clear that the defense reaction of the blood is performed in the field of leucocyte-stimulant factor reaction and in the serum-stimulant factor reaction (antibody-antigen reaction).

As I have mentioned in the report 3[3)], regarding the physico-chemical factors as the antigen in broader sense, production of antibodies in broader sense within the serum is understandable from point of theoretical medicine, in as much as the phases in the hematopoiesis in the bone marrow and the fields of the blood reaction are closely co-operative with each other, so long as the bone marrow is not in insufficiency.

The reason is this. In the adaptation process to the internal environment of the body the tissues (mass of cells) and the humor, leucocytes and serum are in constant relations respectively, the pairs operating antagonistically in the relations. When these relations are broken down, the bone marrow falls into insufficiency, with incapability or inactivity for adaptation to the internal environment. Accordingly, unless the bone marrow and the body are in insufficiency, in leucopenia caused by the second-phase factors of physico-chemical agents, it is clear that a certain substance, antibody, is produced in the serum, that resists the antigen. That is to say, seeing that the bone marrow is irritated and sensitively reacts to the stimulus and that the leucopenia is nothing but an expression of the antibody-antigen reaction, it is impossible to consider that the leucocytes alone react to the stimulant factors while the serum has nothing to do with the factors; it is also impossible to think so, if we see the distribution of the defense energy and the adaptation to the internal environment, so long as the body is not in insufficiency. This will be apprehended more plainly

by recalling the Naegeli[19]'s misleading opinion that leucopenia in typhoid fever has been attributed to the insufficiency of the bone marrow. How can it be so? In fact, the production of the antibodies in the serum of typhoid fever were so much accelerated as to keep the most rational and suitable adaptation to the enternal environment.

When pneumonia, bronchopneumonia or other diseases are complicated in the patient whose leucocytes were remarkably reduced by the usage of X-rays or anticancerous drugs, the leucocytes begin to increase; this fact is an evident indication of a well-arranged adaptation of the body to the internal environment, simultaneously indicating the rise of the production of antibody in the serum, in the humor in broader sense, in inverse relation to the reduction of leucocytes.

Thus, the leucopenia caused by physico-chemical agents is the same with that caused by microbial factors in infectious diseases, and it does not indicate insufficiency of the bone marrow as the leucopenia in typhoid does not indicate such an insufficiency. Yet, it is a matter of course that if these factors operate excessively or far too long periods, the bone marrow either causes organical or functional changes, and falls into insufficiency, or alters into malignant neoplasms.

In infectious diseases, specific antibodies are formed corresponding to the each of infectious factors as antigens. The wellknown antibodies are bacteriotropine, opsonine, bacteriolysine, precipitine, agglutinine, antitoxine, neutralizing antibody and complement fixation antibody.

By the above-mentioned facts, the following assumptions can be taken from the angle of theoretical medicine, though the antibodies in a broader sense that are produced corresponding to those physico-chemical agents as antigens, are not yet practically ascertained:

1) that the production of specific antibodies to the second-phase factors is remarkable, though that to the first-phase factors is slight,

2) that the antibodies are more than half a dozen in kinds,

3) that the antibody in a broader sense lowers the effect of therapy by operating antagonistically to the antigen, when the second-phase factors are put to use for the purpose of medical treatment, for instance, in medical treatment of malignant neoplasms.

Accordingly, the limitation of the effect of therapy by the physico-chemical agents upon malignant neoplasms is here acknowleged. It is therefore reasoned that the more effective manner of treatment is to use these several factors jointly or alternately as to inhibit the rise of antibody production, rather than to use the same factors for long periods.

Biological Significance of "Phases" and "Fields" Observed from the Standpoint of the Internal Environment

In the hematopoiesis in the bone marrow, erythropoietic system holds a

monophasic formation and leucopoietic system holds a diphasic formation; while as for the field of the blood reaction, erythrocytes take one field of O^2 metabolism and leucocytes take two fields in performing the defense reaction in the adaptation process to the internal environment under the constant antagonistic relation with serum, as I have already described. Now the biological significance of the phases and fields requires study from the standpoint of the internal environment in the body.

Claude Bernard[20], the founder of experimental medicine named blood and lymph the milieu intérieur (internal environment) generically, and attached importance to the constant adaptive relation. Also he laid stress on the point that the function of the humor consists not only in the conveyance of nutritions to the cells and the removal of waste products, but also in the presentation of suiable environment to the cells automatically.

All the living creatures have the capacity to alter or to adjust their constitutions and functions for the preservation of their lives, and this capacity is called adaptation. The adaptation is divided into the adaptation to the internal environment and the adaptation to the external environment.

The adaptation to the internal environment is the faculty to keep the relation between the tissues and the humor constant, the faculty to secure the smooth relation among the organs and the faculty to adjust the tissues automatically; while the adaptation to the external environment is the faculty to sustain life coping with the changes of the external world, the external environment.

In this adaptation, the nervous system plays the part of the co-ordinative organ. There are two nervous systems in the mammalia: the autonomous (vegetative) nervous system and the somatic (cerebro-spinal) nervous system; the former governs the adaptation to the internal environment and the latter governs the adaptation to the external environment, presiding over the perception and the movement in the external environment.

It is quite important for the conservation of life to keep the constant relations, — the relation between tissue and humor, and between blood cells and serum — constant.

Now, the adaptation of blood cells is a part of the defense force. The defense force of the blood consists in the defense force of leucocytes and the defense force of serum, and both of them operate antagonistically. The former operates in the field of leucocyte reaction, while the latter operate in the field of serum reaction, both indicating the existence of the two reaction fields. By this two fields of the defense reaction of the blood which take the important post in the process of adaptation to the internal environment, the most sensitive, swift, exact, wide defense reactions are performed to conserve life securely. This function is more easily understood by remembering the said autonomous nervous system as the co-ordinative organ.

There are sympathetic nerves and parasympathetic nerves in the autonomic nervous system, the two operating antagonistically. They govern the adaptation to the internal environment, doubly innervating to the whole organs. At first thought, the double innervations seem to be unnecessary so long as the one system is sufficient to execute the function as the co-ordinative organ. However, in the higher animals such as mammalia, they are so highly specialized organically and functionally and their adapting manners to the environment are so complicated and elaborate that a single system of autonomic nerves as the co-ordinative organ cannot fully execute the adaptative functions and reactions. Therefore, it is necessary and indispensable that the whole organs should be doubly innervated by the sympathetic and parasympathetic nervous systems, in order to preserve life safely.

Thus, in the adaptation to the internal environment in Homo sapiens and in other higher animals, the defense organs such as the blood as well as the co-ordinative organs are carried out securely by a pair of antagonistic systems. By the observance from the standpoint of adaptation to the internal environment in the body, the monophasic formation in the erythropoietic system in the bone marrow and the one field of reaction of the erythrocytes are also easily understood.

The Idea of "Phases" and "Fields" in Hematology and the Theories of Previous Authors

The ideas of "phases" and "fields" have not been thought out as yet in hematology. In studying the principle of hematopoiesis in the bone marrow, the author has found the principle of the one field in the reaction of the erythrocytes and monophasis in the formation of erythrocytes in the erythropoietic system, and the two fields in the reaction of the leucocytes and diphasis in the formation of the leucocytes in the leucopoietic system by introducing the idea of "phases" and "fields" into hematology.

As erythrocytes are formed monophasically in the bone marrow, the mechanism of erythropoiesis is known from old times. Since the appearance of Neumann's[21] report (1868) that the erythrocytes are formed in the bone marrow in every part of the body, a large number of reports have been presented by many authors. Ehrlich[22] (1891) made a new epoch in hematology by various kinds of staining, while Naegeli[23] (1900) systematized the modern hematology on the ground of abundant clinical data.

On the contrary, the principle of leucocyte formation is not clear, various opinions of various schools being voiced without unity. They are the schools of Ehrlich[22] (1891), Naegeli[23] (1900), Pappenheim[24] (1904–1917), Möllendorff[25] (1926) and Sabin[26] (1925) and there are monophyletic, diphyletic or polyphyletic theories in the interrelationship of the blood cells.

As for the genetic mechanism of leucopenia upon which there is much contro-

versy, Markoff[27], Schur and Löwy[28], Faludi[29], Jagic and Klima[30], Barta[31], Arneth[32], Baumann[33] have stated their opinions over the matter and the most prevalent opinion at present is that of Naegeli[19]. Naegeli regarded leucopenia as an expression of insufficiency of the bone marrow in conclusion.

Yet in Naegeli's opinion, there are many contradictions which the author has already pointed out[1-18] and recently Heilmeyer[34] also discredited Naegeli's opinion. Wintrobe[35] states that the pathogenesis of the leucopenia seen as a characteristic of certain infections or of that found in association with overwhelming infections is quite obscure.

In 1940, the genetic mechanism of leucopoiesis was found by Rohr[36], a leading disciple of Naegeli in Zürich University, that was accepted widely as the theory of genetic mechanism of leucocytes.

Although the genetic mechanism of leucocytes was made clearer by this finding, yet it is still obscure; because Rohr, as well as Naegeli, failed to arrive at the ideas of "phases" in the hematopoiesis in the bone marrow and "fields" in the blood reaction.

Furthermore, the genetic mechanism of leucocytes found by Rohr is that of leucocytosis which falls under the first-phase reaction of the diphasic formation in the leucopoietic system in the bone marrow only. Therefore, the genetic mechanism of leucopenia, that is the second-phase reaction of the leucopoietic system in the bone marrow remained in darkness.

The author has expended his main efforts in throwing light on this veiled

TABLE I. Hematopoietic Functions of the Bone Marrow

Hematopoietic functions of the bone marrow	1. Sufficiency	1. Increase in peripheral demands and destruction	Hemolytic and pernicious anemia	Reduction in the blood cell count
		2. Infections and effect of physico-chemical agents	1st phase	Rise of mitosis. Leucocytosis
			2nd phase	Drop of mitosis. Leucopenia
	2. Insufficiency	1. Decrease in peripheral demands and destruction		Inanition
		2. Functional	Nutritional and endocrin disturbances, all types of overwhelming infections, physico-chemical agents if they are given in doses of sufficient size	Beriberi, anemia esp. pernicious anemia, severe cases of sepsis, pneumonia, perityphlitis and others, ionizing radiation such as radium, thorium, uranium and X-rays, chemical agents such as benzol and its derivatives and others.
		3. Organic	Anatomical change	Annihilation or destruction of bone marrow tissues in severe cases of sepsis and in massive dose of physico-chemical agents, metastases of the new growths
	3. Malignant tumors	Myelogenous Leucemia	Acute / Chronic	Widespread proliferation of the myelogenous leucocytes associated with qualitative and quantitative changes

genetic mechanism of leucopenia, in order to inquire into the principle of hematopoiesis in the bone marrow, during these eighteen years of 1940–1958.

Looking to these years, the author keenly feeled the folly of making analysis all the time, forgetting the efforts at synthesizing. That is to say, the author adhered to the hematological changes of the blood cells and was engrossed in studying bacterio-immunity in the clinic of infectious diseases. Owing to this narrow observation, six years was wasted before the idea of "phases" and three years before the idea of "fields" were arrived at. Loitering on the way, however, the author has introduced the idea of phases and fields into hematology, has clarified the principle of hematopoiesis in the bone marrow from the standpoint of the adaptation to the internal environment, has found the fundamentals of the monophasic formation in the erythropoietic system and of the diphasic formation in the leucopoietic system, and has proved them experimentally and numerically.

Table I presents the general view of the hematopoiesis in the bone marrow.

CONCLUSION

The principle of hematopoiesis in the bone marrow depends upon the monophasic formation in the erythropoietic system and the diphasic formation in the leucopoietic system. In the process of adaptation to the internal environment of the body, the erythrocyte reaction takes one field, while the leucocyte reaction, namely, the defense reaction, takes two fields in which leucocytes have constant antagonistic relation to serum.

The monophasic formation in the erythropoietic system and the diphasic formation in the leucopoietic system in the bone marrow are indications of cooperation with the one field of erythrocyte reaction and with the two fields of leucocyte reaction, in adapting to the internal environment.

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Statistical Study on Mortality from Bladder Cancer

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1. *Age-adjusted Death Rates for Bladder Cancer in 20 Countries*

The subject death rates in 1952–56, computed with the figures concerned appearing in the report of WHO[1] as data, and with the total population of 46 countries in 1950 as standard, are shown in Table I. The numbers of deaths are those for the cause listed as Item No. 181 in the Sixth Revision of International Classification of Causes of Deaths, and include the death number for some other urinary organs. In this study, the item is referred to as "bladder cancer" for shortness' sake, instead of "cancer of bladder and other urinary organs".

List of Inclusions (6th Revision)

180 Malignant neoplasm of kidney

Embryoma, kidney	Malignant neoplasm of:
Grawitz's tumour	kidney
Hypernephroma	ureter
	Malignant papilloma of kidney
	Wilms' neoplasm or tumour

180 Malignant neoplasm of bladder and other urinary organs

Malignant neoplasm of:	Malignant neoplasm of:
bladder (sphincter) (urinary)	urethra
urachus	other urinary organs

Dr. Stocks[2] has computed the adjusted death rates for bladder cancer, using the same WHO data, and his published results are also cited in Table I. The ranking is in good agreement with the ours computed with the total population of 46 countries as standard.

Among males, the rate is high in England and Wales, Denmark, Scotland, U. S. Whites, Canada and Australia, the lowest in Japan, and low in Chile, Sweden, Venezuela and Ireland in ascending order. Among females, the rate is the highest in U.S. Non-Whites, next in Canada, U.S. Whites, Northern Ire-

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TABLE I. Age-adjusted Death Rates for Cancer of Bladder and Other Urinary Organs in 20 Countries in 1952–56
(Rate per 100,000 Population. Rates Were Standardized on the Basis of the Age Distribution of the Total Populations of Male and Female in 46 Countries in 1950.)

	Male		Female		Sex ratio	Rate* (computed by Stocks' simple method)	
	Rate	Rank	Rate	Rank		Male	Female
Canada	5.10	5	2.11	2	242	6.47	2.70
Chile[1)]	1.59	20	0.77	20	206	1.98	0.69
U.S.A., White	5.34	4	2.10	3	254	6.77	2.69
U.S.A., Nonwhite	3.92	13	2.57	1	153	4.82	3.16
Venezuela[2)]	2.55	18	0.68	21	375	3.22	0.84
Japan	1.55	21	0.78	19	199	1.96	0.96
Belgium[1)]	4.36	7	1.53	11	285	5.46	1.93
Denmark	5.70	2	1.92	7	297	7.18	2.43
Finland	3.95	12	0.87	18	454	5.01	1.09
France	3.01	16	1.07	15	281	3.79	1.35
Ireland	2.89	17	1.40	14	206	3.58	1.75
Italy	3.56	14	1.02	17	349	4.44	1.29
Norway	3.08	15	1.68	9	183	3.90	2.12
Netherlands	4.25	9	1.79	8	237	5.40	2.28
England and Wales	6.66	1	2.01	5	331	8.39	2.54
Scotland	5.44	3	2.00	6	272	6.82	2.51
Nothern Ireland	4.08	11	2.02	4	202	5.05	2.55
Sweden	2.49	19	1.07	16	233	3.17	1.37
Switzerland	4.21	10	1.45	12	290	5.32	1.83
Australia	4.68	6	1.65	10	284	5.93	2.11
New Zealand	4.29	8	1.42	13	302	5.44	1.80

Note * Quoted from "WHO : Eidem. vital Statist. Rep., 1959, **12**, 222.
1) 1945–56
2) 1952–55

land, and England and Wales in descending order, and the lowest in Venezuela, then Chile, Japan and Finland in ascending order. The highest rate among males (6.66 in England and Wales) is more than quadruple that of Japan (1.55), while among females, the rate among the U. S. Non-Whites (2.57) is only a little less than quadruple that of Venezuela (0.68).

The sex ratio is particularly large in Finland (454), Venezuela (357), Italy (349), England and Wales (331) and New Zealand (302). The lowest ratio occurs in U. S. Non-Whites (153), and also in Norway (183) and Japan (199), it falls below 200.

The adjusted death rates by sex in all the countries are correlated positively at the level of significance of 1%, the countries with high death rates among the

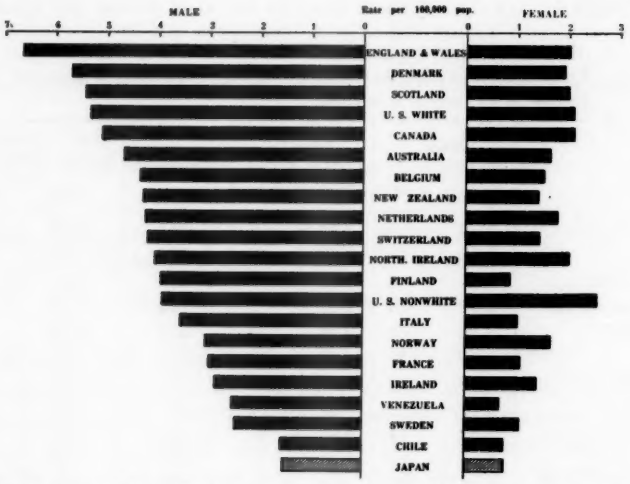

Fig. 1. Age-adjusted death rates for cancer of bladder and other urinary organs in 20 countries in 1952-56.
(Standard population; total populations of 46 countries in 1950.)

male population showing high death rates among the female, in a statistically parallel relation. For reference's sake, the correlation coefficients of the death rates for cancers in some other sites we have computed are shown as follows.

(List No.)	(Site)	(Coefficient)
140 – 148	Buccal cavity and pharynx	+0.336
150	Esophagus	+0.365
151	Stomach	+0.970
152,3	Intestine	+0.971
154	Rectum	+0.952
161	Larynx	+0.121
162,3	Lung	+0.726
181	Bladder and other urinary organs	+0.732
190,1	Skin	+0.889
194	Thyroid gland	+0.926
196,7	Bone and connective tissue	+0.698
204	Leukemia	+0.952

It is seen that the male-female parallelism here is weaker than in the rates for cancers of intestine, stomach, rectum, leukemia and cancers of thyroid gland and skin, but stronger than in those for pulmonary cancer. (No such parallelism was observable in the death rates for cancers of esophagus, buccal cavity and pharynx, and larynx.)

2. *Mortality for Bladder Cancer in Japan*

a. Deaths for bladder cancer in 1947 through 1958

It is since 1947 that data on deaths for bladder cancer have become available. The 5th Revision of International Classification adopted in Japan for 1947–1949 had separate items for "Bladder cancer" and "Cancers of other urinary organs", but in the 6th Revision adopted for 1950–1957, these two items were packed together under one item. The 7th Revision enforced in 1958 again separates them.

In Table II are shown the yearly statistics of death number and crude death rate for bladder cancer for every year and the age-adjusted death rates for every three years since 1947 in Japan, computed with the total population of 46 countries as standard.

TABLE II. Trend of Number of Deaths, Crude Death Rates and Age-adjusted Death Rates for Cancer of Bladder and Other Urinary Organs in Japan (Rates per 100,000 Population. Age-adjusted Death Rates Were Standardized on the Basis of the Age Distribution of the Total Populations of Male and Female in 46 Countries in 1950.)

Year	Male			Female		
	Number	Crude rate	Age-adjusted rate	Number	Crude rate	Age-adjusted rate
1947	236	0.62		175	0.44	
1948	237	0.61	0.95	194	0.47	0.61
1949	275	0.69		231	0.55	
1950	311	0.76		203	0.48	
1951	357	0.86	1.19	218	0.51	0.65
1952	348	0.83		263	0.60	
1953	401	0.94		267	0.60	
1954	509	1.17	1.58	292	0.65	0.76
1955	528	1.20		309	0.68	
1956	554	1.25		352	0.77	
1957	538	1.20	1.77	384	0.83	0.89
1958	625	1.38		352	0.75	

Note Numbers of deaths from "Cancer of bladder" only are as follows:

	1947	1948	1949	1958
Male	215	222	259	612
Female	149	168	210	331

As some cases of cancers of other urinary organs are included as above, the figures in the table may not be called very accurate, but we may see that the age-adjusted death rate for bladder cancer in Japan rose between 1947–49 and 1956–58, by 86% among males and by 47% among females.

The death rates for bladder cancer in Japan in 1947–49, 1950–52, 1953–55, and 1956–58, as itemized by age-classes of 10 years each are shown in Table III. Among males, the rate rises with advance of age, but among women, the rate is the highest in the age-class of 70–79. The rates for men and women are nearly equal up to 40–49, but in the higher age-classes, the difference becomes larger

TABLE III. Trend of Age-specific Death Rates for Cancer of Bladder and Other Urinary Organs in Japan
Rates per 100,000 Population.

Age	1947-49	1950-52	1953-55	1956-58
Male				
All ages	0.64	0.82	1.11	1.28
0- 9	0.01	0.02	0.01	0.03
10-19	0.00	0.02	0.01	0
20-29	0.02	0.01	0.02	0.02
30-39	0.08	0.06	0.10	0.10
40-49	0.41	0.48	0.65	0.57
50-59	1.58	1.90	2.37	2.28
60-69	5.37	6.68	7.66	8.69
70-79	8.93	12.12	18.24	21.62
80+	14.52	14.43	21.59	24.57
Female				
All ages	0.49	0.53	0.64	0.78
0- 9	0	0.01	0.01	0.00
10-19	0.01	0	0.01	0
20-29	0.04	0.02	0·02	0.01
30-39	0.19	0.08	0.10	0.09
40-49	0.56	0.48	0.61	0.58
50-59	1.48	1.60	1.52	1.90
60-69	3.45	3.70	4.16	4.74
70-79	3.83	4.90	7.06	8.33
80+	3.50	4.04	4.03	7.31

with advance of age.

The death rates per age-class from 1947 through 58 in Japan are shown in every three years. We see that the rise in the death rate among males is the higher, the older the age-class. The same tendency is observable also among females, but the rise in recent years among them is gentler than among males.

b. Standardized mortality ratios (SMR) for bladder cancer by prefectures in 1952–1958

We have also computed the SMR for bladder cancer in the individual prefectures in Japan for 1952–1958. I fear the foreign researchers may feel little interest in the figures in the petty prefectures in Japan, and I give only a summary sketch map in Fig. 2. The prefectures with particularly high ratios among males—Kumamoto (153.0), Kyoto (135.0) and Hiroshima (131.5)—all lie in Western Japan. Among females, the ratios are high in Tokyo (135.6), Kyoto (135.2) and Fukuoka (133.0) Prefectures which also lie in Western Japan, except Tokyo.

The prefectures with particularly low ratios both among males and among females are found in Eastern Japan, the ratios tending to be higher in the western prefectures.

The prefectures showing occurrence of patients for schistosomiasis japonica

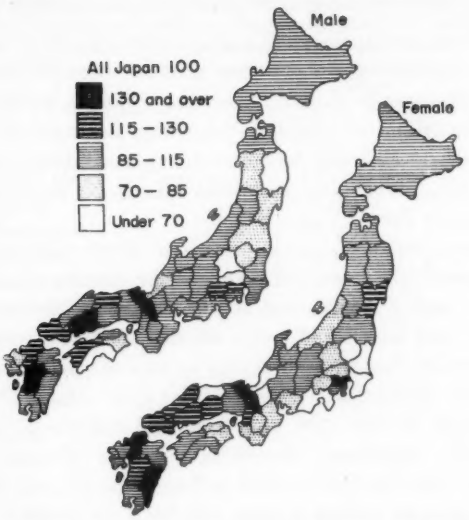

Fig. 2. Standardized mortality ratio for cancer of bladder and other urinary organs by prefectures in Japan in 1952-58.

in Japan are Yamanashi, Saga, Fukuoka and Hiroshima, but in the last, the number of cases is small. The SMR of bladder cancer in Fukuoka is higher than the average of all Japan both among males (126.2) and among females (133.0), in Yamanashi is 119.7 among males and 80.7 among females, and that in Saga is 77.7 among males and 93.8 among females, no inference upon the pathogenic correlation between bladder cancer and schistosomiasis can be made from these figures alone. (Table IV)

TABLE IV. Number of Reported Cases for Schistosomiasis Japonica by Prefectures in Japan, in 1950–1959

Year	All Japan	Yamanashi	Fukuoka	Saga	Hiroshima	Other prefectures
1950	918	643	83	109	76	7
1951	697	458	84	97	45	13
1952	948	359	234	272	77	6
1953	1200	176	456	542	26	0
1954	1537	529	607	395	4	2
1955	1304	356	404	503	37	4
1956	1442	337	776	295	29	5
1957	664	237	234	183	6	4
1958	1983	1183	275	524	0	1
1959	936	584	47	296	5	4

3. *Geographical Correlation between Bladder Cancer and Cancer of Other Sites.*

The geographical correlation coefficients between the death rates for bladder cancer and those for cancers of some other sites were computed for 20 countries and for the 46 prefectures in Japan. There may be some questionable points in the method of calculation we adopted, concerning the standard of rejection of areas with particularly high and low rates, so that we do not claim very high accuracy for the data. With such limitations understood, the results of our computation are given in Tables V and VI.

In the 20 countries table, we see that the death rate for bladder cancer among males is positively correlated with that for cancers of intestine, rectum, lung and prostate and leukemia, and among females, with that for cancers of intestine, rectum, and breast and leukemia, and negatively correlated with the death rate for stomach cancer, both among males and among females.

Next, SMR for bladder cancer and SMR for some other selected sites in the prefectures in Japan in 1954–1956 were computed and the correlation between them was studied. Significant correlation could be observed between SMR for bladder cancer and that for cancers of buccal cavity and pharynx, tongue, and lung among men as well as women, and SMR for bladder cancer and the laryngeal cancer among males. No cases of negative correlation could be found in

Table V. Correlation Coefficients between Age-adjusted Death Rates for Cancer of Bladder and Other Urinary Organs and those for Cancer of Other Sites in 20 Countries, in 1952–56

List No.	Sites	Male		Female	
		Coefficient	Significance	Coefficient	Significance
140-148	Buccal cavity and pharynx	+0.309		−0.106	
150	Esophagus	−0.212		−0.314	
151	Stomach	−0.564	−−	−0.657	−−
152, 153	Intestine	+0.753	++	+0.725	++
154	Rectum	+0.747	++	+0.678	++
161	Larynx	−0.070		−0.299	
162, 163	Lung	+0.719	++	+0.389	
170	Breast	.	.	+0.781	++
171-174	Uterus	.	.	−0.201	
177	Prostate	+0.528	+	.	.
190, 191	Skin	+0.293		−0.064	
941	Thyroid gland	+0.199		+0.198	
196, 197	Bone and connective tissue	+0.072		−0.358	
204	Leukemia	+0.584	++	+0.441	+

Note + Significant positive correlation at the level of 5%.
++ Significant positive correlation at the level of 1%.
−− Significant negative correlation at the level of 1%.

TABLE VI. Correlation Coefficients between Standardized Mortality Ratios for Cancer of Bladder and Other Urinary Organs and Those for Cancer of Other Sites by Prefectures in Japan, in 1954–56

List no.	Sites	Male		Female	
		Coefficient	Significance	Coefficient	Significance
140-148	Buccal cavity and pharynx	+0.342	+	+0.388	++
141	Tongue	+0.299	+	+0.326	+
150	Esophagus	+0.103		−0.131	
151	Stomach	−0.123		+0.038	
152, 153	Intestine	+0.188		+0.038	
154	Rectum	+0.281		+0.111	
155, 156	Liver and biliary passages	+0.087		−0.054	
157	Pancreas	+0.002		+0.279	
161	Larynx	+0.305	+	+0.284	
162, 163	Lung	+0.417	++	+0.322	+
170	Breast	.		+0.112	
171-174	Uterus	.		+0.150	
175. a	Ovary	.		+0.148	
177	Prostate	+0.072		.	
190, 191	Skin	−0.040		+0.255	
196	Bone	+0.162		+0.195	
204	Leukemia	+0.114		−0.039	

Note + Significant positive correlation at the level of 5%.
++ Significant positive correlation at the level of 1%.

this study.

In all these studies on correlation, the significant correlation between bladder cancer and the lung cancer was notable. Though in the death rates for bladder cancer and for lung cancer among females in the 20 countries, no significant positive correlation could be observed, in all the other cases (among males in the 20 countries and among males and females in the Japanese prefectures), a significant positive correlation between the two sets of death rates could not be denied.

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On the Essential Nature of Hematopoietic Function of Bone Marrow

Report 5: On the Leucocyte Reaction in Infectious Diseases with Special Reference to Schilling's Biological Leucocyte Curve

By

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(Received for publication, March 26, 1961)

As reported in the four preceding papers[1-4], the present author has been convinced that the essential nature of hematopoietic function of the bone marrow lies in the monophasic formation of erythrocytes and the diphasic formation of leucocytes, and that these one–two phases correspond to the single-field reaction of erythrocytes and the double-field reaction of leucocytes, thus contributing to the maintenance of life by adapting the living organism to the internal environment in the most rational and most purposeful way possible[5-27].

The blood consists of blood cells and serum, which stand in constant antagonistic relation in the drama of adaptation to the internal environment, the effect being reflected in the peripheral blood in the forms of leucocytosis, normo-leucocytosis and leucopenia. It is, therefore, meaningless to speak on the essential nature of leucocyte reaction without paying due attention to the mutual relation of blood cells and blood plasma or serum.

Observation on Leucocyte Reaction from the Standpoint of Peripheral Blood

When viewed from the angle of the fields of blood reaction, leucocytosis and leucopenia show essential difference between them, provided that the bone marrow function is sufficiently maintained, for in the former, the vital defense reaction is deployed in the field of cell-bacterium reaction with neutrophils as the main defense force, while in the latter, the main battle field is in the field of antibody-antigen reaction, the leading role being taken by lymphocytes.

A. Leucocyte Reaction of Leucocytotic Type

Leucocyte reaction occurs in the form of leucocytosis in the following infections:

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1) Acute appendicitis: For example, the change in the peripheral blood picture in a patient of 17 years, male, school boy, is given in Fig. 1.

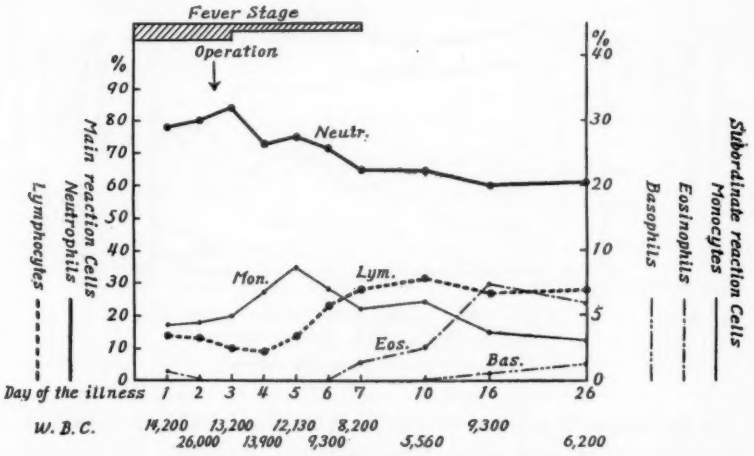

Fig. 1. Acute appendicitis, school boy aged 17.

On the 1st day of disease, malaise, fatigue, nausea, poor appetite and ileocecal pain were complained of, temperature rose to 38.3°C, the total leucocyte count to 14,200, the ratio of neutrophils to 78%, nuclear shift to the left was prominent; on the 2nd day, temperature stood at 37.8°C in the morning and the abdominal pain was somewhat alleviated, but in the evening, temperature rose to 39.6°C, the leucocyte count sprang up to 26,000, and preparations were made for operation, but owing to reasons in the patient's family, it was postponed; on the 3rd day, appendectomy was performed and findings of appendicitis gangraenosa were revealed. On the 16th day, the patient was dismissed as completely cured.

In this case, the change in the leucocyte picture consisted in increase of neutrophils, decrease of lymphocytes and eosinophils and disappearance of basophils down to the 3rd day, decrease of neutrophils and increase of lymphocytes, especially of monocytes on the 4th — 7th day, and increase of lymphocytes, especially marked increase of eosinophils, and reappearance of basophils.

2) Scarlet fever: The change in peripheral leucocyte picture traced in 38 cases of the disease was as shown in Fig. 2. In these cases, temperature keeps a relatively high level down to the 5th day of disease, but then begins to come down, usually to the normal by the 7th—9th day, but in some of the cases, fever lasted till the 12th day.

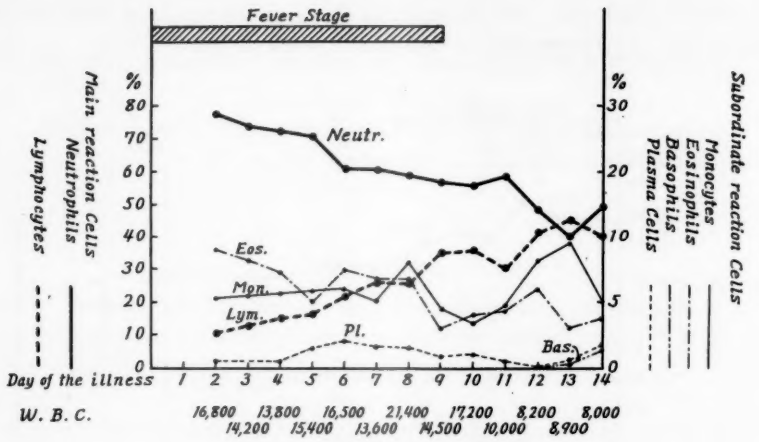

Fig. 2. Scarlet fever (38 cases).

In the leucocyte picture, neutrophils were found highly increased in the stadia of rising-fever and acme, eosinophils also increased, but lymphocytes were decreased and basophils disappeared from the peripheral blood; in the fever-abating stage, monocytes increased, neutrophils decreased and lymphocytes and plasma cells increased; in the convalescent stage of the 12th — 14th day, lymphocytes were markedly increased, neutrophils were decreased and basophils made reappearance, followed by a picture of post-infectious basophilia.

3) Eruptive typhus: In this disease, the period of high temperature lasts 10–14 days, averaging 12 days. The leucocyte count shows the mean value of 14,600 in the 1st week of the disease, but turns lower thereafter toward the normal level.

In the incipient and the acme stages, neutrophils increase rapidly, nuclear left shift is marked and the decrease of lymphocytes, eosinophils and basophils become obvious; in the fever-abating stage, plasma cells increase markedly and monocytes also increase but neutrophils decrease, the increase of lymphocytes becoming apparent; in the reconvalescent stage, lymphocytes increase more and more, to attain the maximum count in the IVth week, but fell to the normol level thereafter, eosinophils run down to the lowest level in the IInd week of the disease but increase thereafter, to cause a post-infectious eosinophilia of 5% on the average in the 5th—6th week, the plasma cells appeared in the average percentage of 0.7% in the peripheral blood even in the same period, while monocytes increased further before falling to the normal level after the convalescence.

4) Relapsing fever: The change in leucocyte picture was traced in the typical second fever attack in a woman of 27 years who had the disease as com-

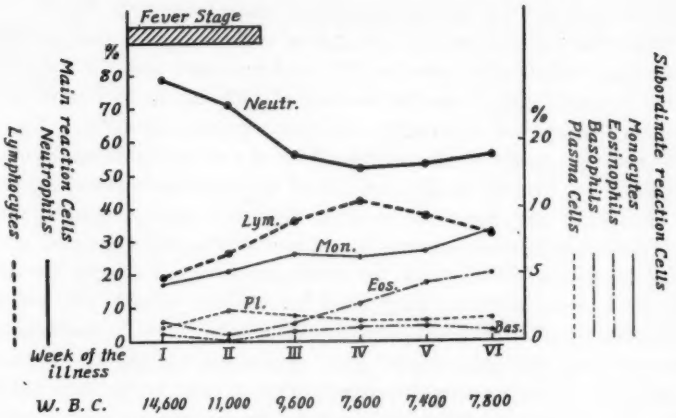

Fig. 3. Eruptive typhus (142 cases).

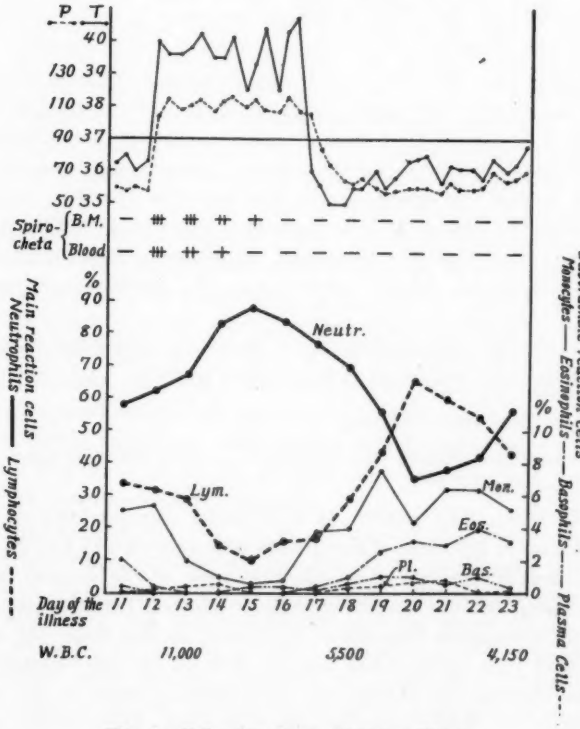

Fig. 4. Relapsing fever, 27 years old, ♀.

plication of typhoid fever in the convalescent stage is given in Fig. 4.

Fever broke out first on the 12th day of typhoid fever, accompanying chill and shivering, temperature rose to 41°C and remained high for 5 consecutive days, but dropped critically in the morning of 17th day to 35°C with sweating, showed hypothermia for a few days and then regained normalcy.

The leucocyte count in the peripheral blood rose to 11,000 on the 12th day of typhoid fever, namely on the 2nd day of typical relapsing fever; in the first stage of attack and acme neutrophils increased rapidly, lymphocytes and monocytes decreased, while eosinophils and basophils could be detected no more; with the cessation of fever attack, the monocytes increased steeply, the decrease of neutrophils and the relative increase of lymphocytes became quite perceptible and the tendency of increase appeared in plasma cells; in the convalescent stage, lymphocytes increased ever more, their percentage running up to 65.5% in the maximum, in wide excess over the 24.7% of neutrophils, then the former began to fall off and the latter to come up, eventually to regain normalcy, monocytes and eosinophils attained the highest percentages of 6.6% and 5.4% on the 19th and 22nd day of disease, respectively, and then normalized. The other 7 cases of the disease I examined showed similar courses.

In relapsing fever, the temperature suddenly jumps up from the normal to the high of around 40°C and equally suddenly drops to the normal or even lower after a stage of continued fever for 5—7 days, so that the disease furnishes data well-adapted for studying the relation between pyrexia and leucocyte reaction. The present author has examined the hematopoietic function, especially the mitosis of nucleated cells, in the bone marrows of 8 relapsing fever cases at 4 days' intervals, and found that the mechanism of leucocyte formation in the bone marrows in leucocytosis actually agreed with the description as reported by Rohr[28].

Also in Japanese encephalitis, acute pneumonia and some other diseases, the leucocyte reaction was found to take similar courses.

B. Leucocyte Reaction of Leucopenic Type

The leucocyte reaction of this type shows a quite different form that in the leucocytotic type above.

I) Typhoid fever: The results of 1727 serial examinations of peripheral leucocyte picture in 536 cases of the disease were as shown in Fig. 5.

In the first fever-rising stage (the first week of the disease), neutrophils increase, bringing out the symptomatic leucocytosis ("Verteilungsleukocytose") as one of the elementary formulae of infections; in this stage, the mean total leucocyte count runs up to 9,200, nuclear shift to the left becomes apparent, lymphocytes decrease to 14.3% but neutrophils increase to the excessively high percentage of 83.6%; in the acme (the IInd—IIIrd week), neutrophils

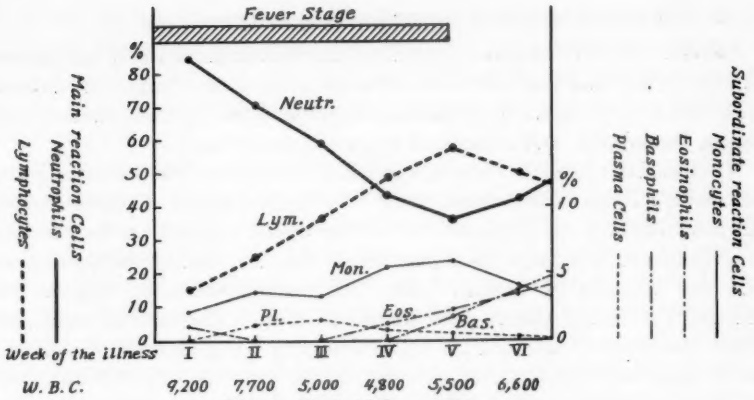

Fig. 5. Typhoid fever (536 cases).

decrease steeply to the normal level of 50–60 % and consequently leucocyte counts become normal (6,000–9,000), lymphocytes increase relatively and then absolutely, but monocytes remain in the normal range, while eosinophils and basophils decrease, in most cases to nil, and plasma cells that came into appearance in the fever-rising stage increase to 1.9% ; with progress of the disease, neutrophils decrease further but lymphocytes keep on increasing, so that their curves cross each other, forming the so-called typhoid cross, in most cases the total count of leucocytes falling below 5,000, and in moderate or milder cases eosinophils reappear in the IIIrd disease week ; in the indeterminate fever stage and the fever-abating stage (the IVth—Vth week), the total leucocyte count drops to 4,890 on the average (1,600 in minimal cases), in particular the decrease of neutrophils is conspicuous (to 36.3% on the average, to 8% in severe cases and to 128 in absolute count), while lymphocytes increase relatively to 56% on the average and to 90% in severe cases ; in the reconvalescent stage (in and after the Vth—IXth week), the total leucocyte count gradually increase, neutrophils increase and lymphocytes decrease, so that their curves cross again and normalcy is slowly reinstated, while plasma cells disappear from the peripheral blood, monocytes decrease to the normal range, bringing out the picture of post-infectious eosinophilia and basophilia.

2) Paratyphoid A and B : As in the preceding disease, leucopenia was induced by decrease of neutrophils, but the degree of decrease was milder in paratyphoid A and more so in paratyphoid B. The double crossing of the curves of neutrophils and lymphocytes was also the widest-spaced in typhoid fever, less so in paratyphoid A and the narrowest-spaced in paratyphoid B. Such a gradation in the decrease of leucocytes and the space between the double crossing well corresponds to the severity of clinical symptoms in these diseases.

C. Leucocyte Reaction of Normo-leucocytotic Type

In some infectious diseases, including infectious mononucleosis, tuberculosis and leprosy, the total leucocyte count remains in the normal range, the decrease of neutrophils being made up by increase of lymphocytes. An intermediate type between leucocytotic and leucopenic types is present here.

I) Glandular fever: The case was a man of 26 years, who had immigrated from western Japan in September, 1944. On the first attack of disease, he felt chill, head-ache and rise of temperature to 38.8°C, and to 39.4°C on the next day, the level around 39°C being maintained till the 4th day, and he was quarantined under the diagnosis of typhoid fever. At hospitalization, the pharynx was reddened, the lymph glands were tumefied, those in the cervical region, the axillae, the cubital fossae and the inguinal regions being found swollen to pea-size or small-finger tip-size, the enlarged spleen could be palpated, no roseola was observed, Widal's test showed negative result, diazo reaction test also gave negative result, and cultural findings of typhoid bacilli from bone-marrow, blood, stool, urine, pharyngeal mucus, and bile failed, too. After hospitalization, high temperature of 37.6°–39°C persisted till the 19th day, and the patient was dismissed as healed on the 33rd day of disease.

In this disease, the total leucocyte count remains nearly within the normal range, as shown in Fig. 6, the decrease of neutrophils being made up by the increase of lymphocytes, but in particular, the curves of these two types of cells come to the first crossing on the 11th day, neutrophils fell to the lowest percentage of 24.2% and lymphocytes attained the highest percentage of 70.4% on the 14th day of disease, and yet the total leucocyte count stood at the normal value of 8,600; thereafter, neutrophils increased and lymphocytes

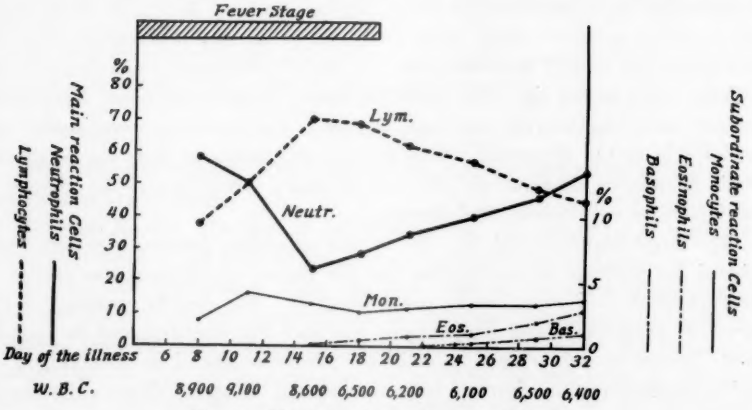

Fig. 6. Glandular fever, 26 years old, ♂.

decreased, so that the second crossing of their curves fell on the 29th day and gradually the normal proportion was recovered, while monocytes attained the highest level of 6.9% on the 18th day, eosinophils and basophils increased in the convalescent stage and indicated presence of post-infectious eosinophilia and basophilia.

2) Tuberculosis: In the incipient stage, the reserved neutrophils in the bone marrow are mobilized in symptomatic leucocytosis by the demand from the periphery, and then by the intensification of mitosis of the neutropoietic system in the bone marrow the total leucocyte count rises to 12,000–18,000 and even beyond 20,000 in some cases. But as the tuberculous germs belong to bacilli and the reaction angle of neutropoietic system in the bone marrow is small, standing between the first- and the second-phase factors in type, advanced leucocytosis never comes forth in tuberculosis, unless first-phase factors come in mixed infection: with further progress to the better, neutrophils begin to decrease, but the decrement is made up by the increment of lymphocytes and the leucocyte count remains normal; so, during exudative process or relapse in the chronic course of disease, monocytes first begin to increase and then also neutrophils, and improvement in these conditions brings about increase of lymphocytes, eosinophils and basophils, the main arena of defense reaction shifting to and fro between the Ist and the IIIrd region.

Schilling's Biological Leucocyte Curve

Attention was directed to the problem of changes of leucocytes in infectious diseases by Ziegler & Schlecht[29], Arneth[30] and some others already, but we must mention Schilling[31] as the author who has gone most deeply in the question of the blood picture in infectious diseases. He examined the change of leucocytes in the course of infectious diseases and described a leucocyte curve which consisted

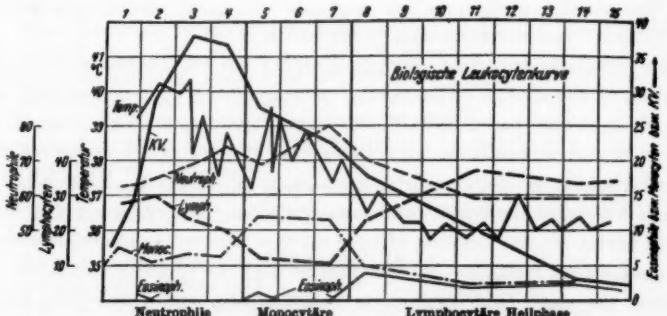

Abb. 266. Verschiebungen des weißen Blutbildes beim akuten Infekt. (Nach V. SCHILLING.)

Fig. 7. Schilling's biological leucocyte curve.

of three parts called by him the neutrophilic struggle phase (Neutrophile Kampfphase), the monocytic defense phase (Monocytäre Überwindungsphase) and the lymphocytic healing phase (Lymphocytäre Heilphase).

In Fig. 7. is reproduced the curve well-known as the Schilling's biological leucocyte curve ("Biologische Leukocytenkurve"). This curve was originally obtained upon examination of a woman of 23 years who suffered from post-puerperal sepsis, complicated with mastitis, iritis and then with angina, accompanying pyrexia, enlargement of spleen and erythema nodosum.

In this case, fever rose to 40°C on the 3rd disease day by acute aggravation of sepsis, the blood showed a strong leucocyte reaction, metamyelocytes and rod-shaped neutrophils reaching 36% of total leucocytes and nuclear left shift being obvious, and the formation of neutrophils was highly intensified to meet the great consumption of them in the periphery, revealing a picture of enhanced demand and proportionate increase of leucocytes; on the 5th day of disease, the nuclear left shift ceased to be observed, a transient increase of leucocytes appeared on the 7th day and normalcy was recovered on the 13th day.

From such findings, Schilling arrived at his tripartite course of infectious diseases cited above.

The first phase of neutrophilic struggle:
Leucocytosis and nuclear left shift are apparent, and decrease of lymphocytes and rarefaction of eosinophils, sometimes to nil, occur as specific features.

The second phase of monocytic defense:

Rivival of pyrexia occured on the 5th—7th day, erythema nodosum appeared and monocytes increased to 6–12%. Schilling opines that such symptoms are induced by wide-scoped mobilization of defense substances aiming at critical alleviation of the disease by means of the reticuloendothelial system.

The third phase of lymphocytic healing with post-infectious eosinophilia:

In this stage, lymphocytes are markedly increased, producing a picutre of post-infectious lymphocytosis and eosinophilia.

Since the above tripartite curve of Schilling has been obtained by empirical observation of the change of leucocytes of leucocytotic type disease and not by pursuit of the change based upon the leucocyte function, cases are not rare where the curve is not reconcilable with the actual picture of leucocyte reaction in infectious diseases, as Heilymeyer[32] has already pointed out. Especially, as the Schilling's biological leucocyte curve was traced in the days when the essential nature of hematopoietic function of bone marrow was yet unknown, not to speak of the monophasic formation of erythrocytes and the diphasic formation in the leucocytes, the single-field erythrocyte reaction and the double-field leucocyte reaction, it is easy to conjecture that this curve has failed to reflect the leucocyte reaction in infectious diseases with accuracy.

In fact, the leucocyte reaction in infectious diseases, as represented in Figs.

1–6, have been found to show uniform basal forms, according to the three types of leucocytotic, normo-leucocytotic and leucopenic variations.

The Schilling's biological leucocyte curve and his names of the three phases in it are reconcilable with the findings in my leucocytotic type diseases, but not with the courses of my normo-leucocytotic and leucopenic type diseases.

CONCLUSION

The reaction behavior of leucocytes in infectious diseases shows the three different but respectively definite forms of leucocytotic type, normo-leucocytotic type and leucopenic type.

The Schilling's biological leucocyte curve is applicable to the cases of my leucocytotic type, but not at all to the cases of my normo-leucocytotic and leucopenic types.

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On the Essential Nature of Hematopoietic Function of Bone Marrow

Report 6: Observations on the Leucocyte Reaction from the Standpoint of the Fields of Blood Defense Reaction with Special Reference to the Three Basal Forms of Leucocyte Reaction

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(Received for publication, March 26, 1961)

The changes of leucocytes observed in infectious diseases, as stated in the previous report[1], show very regular differences in leucocytosis, normoleucocytosis and leucopenia.

These differences are brought forth by the different intensity of cell-bacterium reaction and antibody-antigen reaction in the first, second and third regions of the fields of blood defence reaction.

Here, the cell-bacterium reaction and the antibody-antigen reaction are essentially different, show inversely related intensity, act antagonistically to each other, and the selection of the main field of defense reaction out of the three regions depends on the specificity of the pathogenic agent.

Accordingly, from the veiw point of adaptation to the internal environment we have to study the leucocyte reaction in the cases of leucocytosis, normoleucocytosis and leucopenia in infectious diseases[2-28].

Leucocyte Reaction as Viewed from the Angle of Fields of Blood Defense Reaction

The relationship between the fields of blood defense reaction and the leucocyte reaction is as shown in Fig. 1.

1. Leucocyte Reaction in Leucocytosis

(Main Defense Reaction Takes Place in the First Region)

The arena of main defense reaction of leucocytosis lies in the Ist region where the cell-bacterium reaction is in predominance. The leucocyte reaction in this region has the main feature in leucocytosis, the leucocyte count being increased

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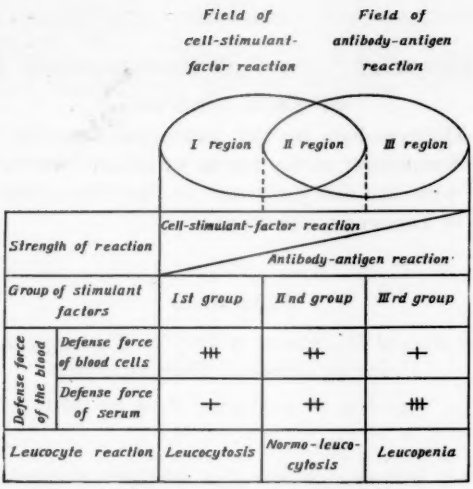

Strength of reaction	Cell-stimulant-factor reaction / Antibody-antigen reaction		
Group of stimulant factors	Ist group	IInd group	IIIrd group
Defense force of the blood — Defense force of blood cells	+++	++	+
Defense force of the blood — Defense force of serum	+	++	+++
Leucocyte reaction	Leucocytosis	Normo-leucocytosis	Leucopenia

Fig. 1. Defence reaction fields of the blood.

by neutrophilia. In infections by streptococci, staphylococci, diplococci, and in local infections including bacillary dysentery, hemorrhagic, purulent and spasmodic diseases, the main defense reaction of blood is deployed in the Ist region.

In such a leucocyte reaction in the Ist region, the peripheral demand for leucocytes rises with the outbreake of disease, the potential-falling gradient between the bone marrow and the peripheral blood steepens abruptly, and the reserved neutrophils in the bone marrow wander out into the peripheral blood, resulting in decrease of nucleated cells and a nuclear shift to the left in the bone marrow and leucocytosis due to increase of neutrophil cells and a nuclear shift to the left in the peripheral blood.

Such a change in leucocyte distribution at the first stage of disease, being due not to a change in the hematopoietic function of the bone marrow but to a change in the distribution of neutrophils caused by a steepening of the potential-falling gradient between the bone marrow and blood, represents a distribution or symptomatic leucocytosis. Thus the changes in the bone marrow should be called symptomatic decrease of the nucleated cells in the bone marrow and a symptomatic nuclear shift to the left, and those in the peripheral blood symptomatic leucocytosis and a symptomatic nuclear shift to the left, respectively.

If the peripheral demand for blood cells is the heavier, the occurrence more abrupt and the grade of disease severer, the symptomatic leucocytosis in the peripheral blood comes forth very intense in such cases, on the 1st—3rd days of disease.

On the other hand, the bone marrow comes to show an intensified mitosis in the neutrophilic series by the stimulus of the first-phase factor, and shows the first-phase reaction ; the cells of neutrophilic series are markedly proliferated, so that their consumption and destruction in the peripheral blood is more than sufficiently made up, and many matured cells of the neutrophilic series, much more than in normal state, are contained in the bone marrow (Figs. 2 and 3).

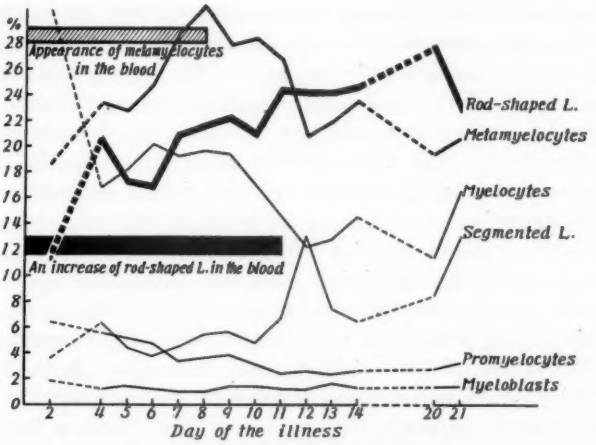

Fig. 2. Change of the neutrophil cells in the bone marrow of the patients with leucocytosis.

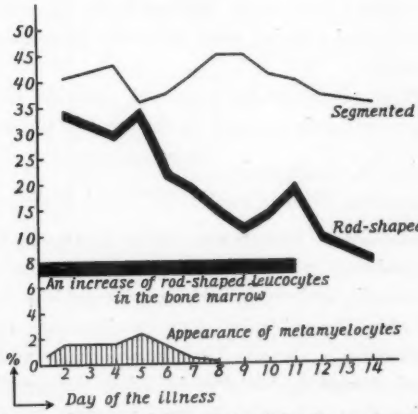

Fig. 3. Change of the neutrophil cells in the peripheral blood of the patients with leucocytosis.

From this fever-rising stage down to the first stage of acme, the blood defense reaction is enacted in the Ist region in Fig. 1, with neutrophils as the leading actor, and the leucocyte reaction shown in the preceding report (Report 5) is observable. This stage corresponds to the Schilling's[39] so-called struggle phase ("Kampfphase").

In the fever-abating stage, the infecting germs are gradually overpowered by the mighty defense force of the host and decimated microbes are left in forlorn in the sporadically remnant centers. In this stage, the fever becomes intermittent and an irregular curve is described. Neutrophils decrease steadly while monocytes increase notably and lymphocytes less notably, and the once decreased or disappeared eosinophils and basophils tend to reappear or to reincrease, but the total leucocyte count is gradually reduced by the large decrease of neutrophils. The field of main defense reaction shifts over to the IInd region (Fig. 1) and shows the stage of Schilling's so-called defense phase ("Abwehr- oder Überwindungsphase").

Next, in the reconvalescent stage, the pathogenetic microbes are annihilated, fever abates completely, lymphocytes increase more than ever, eosinophils and basophils also increase, showing the picture of post-infectious eosinophilia and post-infectious basophilia, while neutrophils and monocytes lose in number. In this stage, the main defense reaction takes place in the IIIrd region (Fig. 1), and the Schilling's so-called lymphocytic healing phase with post-infectious eosinophilia (Lymphocytäre Heilphase mit postinfektiöser Eosinophilie) is presented.

In the bone marrow, overripened neutrophils and eosinophils make appearance in the fever-abating stage, representing inhibited or quiescent type emerging after stimulation; such types appeared also in the peripheral blood 4–7 days later, in the period ranging from the later fever-abating stage to the early convalescent stage.

In severe cases of leucocytosis, the leucocytic reaction in the Ist region is protracted, and the cases of death occurred in the struggle phase in the Ist region.

2. Leucocyte Reaction in Leucopenia
(Main Defense Reaction Takes Place in the Third Region)

The main arena of defense reaction resulting in leucopenia is laid in the IIIrd region where the antibody-antigen reaction is prevalent. The leucocyte reaction in this region is enacted with lymphocytes playing the leading role, and leucopenia is brought about by reduction of neutrophil count, but the production of antibodies by lymphocytes is intensified in inverse relation and the strengthened antibody-antigen reaction bears the brunt of defending the organism.

As a representative case of leucopenia type reaction, the leucocyte reaction in typhoid fever is described in following.

In the first stage of typhoid fever, the demand for neutrophils from the periphery is strengthened, the potential-falling gradient between the bone marrow and the peripheral blood is steepened, and as the result, the mature neutrophils reserved in the bone marrow flow out into the peripheral blood, symptomatic decrease of the nucleated cells and nuclear shift to the left occur in the bone marrow, while symptomatic leucocytosis and nuclear shift to the left occur in the peripheral blood (Figs. 4 and 5); so, the peripheral leucocyte count rises to 8,000–15,000, and leucocytosis of average count of 9,200 is present. This stage corresponds to the fever-rising stage and the reaction takes place in the Ist region.

With the progress of disease, the leucocytes are decreased; neutrophils are most prominently decreased, while eosinophils and basophils are also decreased, to nothing in most cases. On the other hand, lymphocytes increase relatively, showing the normal leucocyte count of 7,700 in the second week of disease. In this stage, the main defense reaction takes place in the IInd region (Fig. 1). The titer of antibodies rises in inverse relation to the decreasing leucocyte count, the agglutination titer in Widal's reaction becomes positive (above $\times 200$), the cell-bacterium reaction comes to equilibrium with the antibody-antigen reaction, and clinically the first half of the acme is reached, the typhoid symptoms becoming more and more apparent. In the latter half of acme,

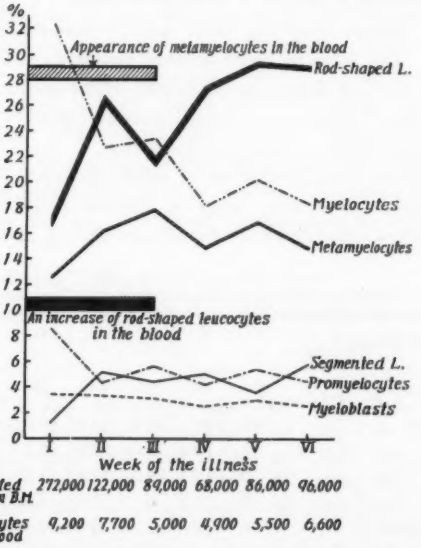

Fig. 4. Change of the neutrophil cells in the bone marrow of the patients with leucopenia.

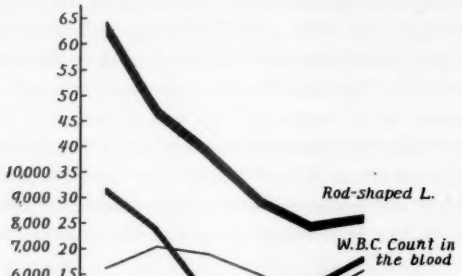

Fig. 5. Change of the neutrophil cells in the peripheral blood of the patients with leucopenia.

leucopenia is intensified owing to further decrease of neutrophils, plasma cells and monocytes increase, and the main defense reaction shifts to the IIIrd region in Fig. 1. The titer of agglutination reflecting the output of antibodies begins to rise on the 5th day or so of disease, turns positive, rising above $\times 200$ on the 6th–8th day of disease, and keeps on rising linearly thereafter, to reach the highest level in the IIIrd—VIth week of disease.

In typhoid fever, the production of antibodies rises inversely to the fall of neutrophil formation, the main energy of defense reaction being mobilized for antibody production, the typhoid bacilli circulating over the whole body are

Table I. The Relation between the Fortune of Typhoid Bacilli and

Course of the illness	1 day	2	3	4	5	6	7	I week	II	III
No. of cases		1	9	12	14	22	53	111	356	403
Bone Marrow		100	100	83.3	92.9	90.9	92.5	91.9	81.2	69.0
Blood		0	78.8	75.0	92.9	77.3	73.6	76.6	47.8	31.5
Stool		0	11.1	33.3	21.4	31.8	35.9	30.6	34.0	40.0
Urine		0	11.1	0	7.2	4.6	5.7	5.1	4.8	8.9
Pharyngeal mucus		0	0	0	0	0	1.9	0.9	1.1	3.2
Widal's reaction more than × 200		—	—	—	29	45	72	72	84	89

taken up by biochemical substance, which deprives the bacilli of their freedom, damages or blocks their metabolic mechanism and kills them by such antibiotic action. Such an antibiotic action begins to be manifest on the 5th day of disease, so that, as shown in Table I, the cultural finding rate of the bacilli falls off rapidly after attaining the maximum of 92,9% on the 5th disease day, to 47.8% in the IInd week of disease, and the detection of the bacilli in circulating blood becomes difficult. On the other hand, though the bacilli may succeed in establishing foci well-adapted for their multiplication in this week, the bacilli are not yet excreted in not rare cases, so that the cultural finding for them in stool and urine show negative results and the titer of Widal's reaction remains unrisen. Baserga and Barbagallo[30] have named this week the silent stadium ("Stummes Stadium").

Since, however, the bone marrow is rich in capillary vessels, the blood flow in them being slow, belong to the reticuloendothelial system and have so many apparatus for screening and arresting pathogenetic microbes in the capillary walls, a high percentage of the positive bone marrow culture is observable here even in the very early stage of typhoid fever. In the IIIrd week, the proliferation foci become the souces of excreted bacilli, which are now detected in stool, urine, breast milk, bile, etc; the gall bladder offering a particularly well-adapted center of bacillar multiplication becomes a foremost source of separation, showing a high detection rate.

The IIIrd and IVth weeks are the period of most desperate host-parasite fight, pneumonia, intestinal hemorrhage, intestinal perforation, decubitus, bradyacusia and such complication are apt to come in during these weeks, and the host is exposed to highest danger; death of severe cases also supervene most frequently in this period. The agglutination titer also becomes strongly positive in this stage, and indeed a case with ×51,200 titer was experienced. On the other hand, neutrophils are found decreased, the leucocyte count falls to 4,900 on the average, the mean percentage of lymphocytes rises to 56%, in some severe

Widal's Reaction (More Than ×200) in 536 Typhoid Cases

IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	Total
321	214	129	81	43	25	17	8	5	6	4	4	1,727
54.5	49.5	45.0	37.0	44.2	48.0	58.8	62.5	80	66.7	75.0	0	63.4
19.9	15.9	10.9	12.4	9.3	20.0	5.9	25.0	20	0	0	0	29.9
30.2	26.2	24.8	19.8	20.9	24.0	52.9	12.5	20	33.3	75.0	25.0	31.8
11.2	12.6	21.7	28.4	30.2	24.0	35.3	12.5	20	50.0	25.0	0	11.8
3.1	4.2	3.1	2.5	2.3	0	5.9	12.5	20	16.7	25.0	25.0	2.9
92	95	93	92	90	88	85						

cases reaching 80–90%, but that of neutrophils fell to only 8% in some of them.

In the reconvalescent stage in the Vth—VIth weeks, the leucocyte count gradually regain the normal value through increase of neutrophils and decrease of lymphocytes, eosinophils and basophils also increase and the pictures of post-infectious eosinophilia and basophilia become apparent.

Thus, the number of neutrophils that determine the total leucocyte count decrease widely in the IIIrd—VIth weeks while the antibody value rises in the same period, showing a vivid negative correlation.

Accordingly, the main leucocytic defense reaction in leucopenic infections is played out in the Ist region during the fever-rising stage, in the IInd region in the first half of acme and in the IIIrd region in the subsequent stages. The crucial host-parasite battle is fought in the IIIrd region, with lymphocytes as the leading defense force.

3. Leucocyte Reaction in Normo-leucocytosis
(Main Defense Reaction Takes Place in the Second Region)

In tuberculosis, leprosy, syphilis and some other bacillary infections, the total leucocyte count remains in the normal range of 6,000–9,000, the decrease of neutrophils being made up by increase of lymphocytes, the cell-bacterium reaction is on the same footing with the antibody-antigen reaction and the main defense reaction is carried out in the IInd region.

For example, in tuberculosis, symptomatic leucocytosis first makes appearance in the initial stage when the antibody formation is not yet begun, then the leucocytosis is shown by more frequent mitosis in the leucopoiesis of the bone marrow, sometimes to the level of 12,000–19,000 of total leucocyte count. In this stage, the leucocytes fight their battle between host and parasites in the Ist region with the fast-proliferating neutrophils as mainstay.

With further progress of the disease, neutrophils begin to decrease and lymphocytes begin to increase, the total leucocyte count returns to the normal level, and in the cases of cirrhotic pulmonary tuberculosis and tuberculosis running a favorable course, lymphocytes increase more markedly, while in the cases of aggravating disease, the increase of neutrophils and the nuclear shift to the left become prominent, but when pyrexia remains mild, the increase of neutrophils is also mild, and only slight leucocytosis induced, even in the cases with enlarging foci. In the severe cases, the leucocytosis aggrevates sometimes to the level of around in 20,000 leucocyte count, but even in such cases, the increase of total leucocyte count is usually smaller than in the cases of coccal infection.

When the agonal stage is approached, leucocytes decrease and leucopenia is induced, sometimes sudden and violent fall of the leucocyte count (the so-called "Leukocytensturz") setting in. This leucopenia is due to insufficiency of the

bone marrow function.

Tuberculous bacilli take about one month for growth in vitro culture, or about 40 times of the term (18 hours) required for similar culture of typhoid bacilli; in vivo, the total course of tuberculosis also takes about 40 times as long a time as that for recovery from typhoid fever (3 years against 4–6 weeks). Thus, tuberculous bacilli take much more chronic courses for growing in vitro as well as infecting in vivo than typhoid bacilli. During these long courses, the former often induce exacerbation in the patients, the progress of disease taking zigzag form ; on such occasions, the IInd region becomes the scene of the main defense reaction, which is often drawn over and over a long period — a feature of specifying the clinical course of tuberculosis.

Even when the main reaction arena lies equally in the IInd region, remarkable changes in differentials of white blood cells and in clinical symptoms will appear in the case when the main reaction arena is nearer to the Ist region, from those in the case when the main reaction arena lies nearer to the IIIrd region. The more the main reaction arena is shifted to the side of Ist region, the increase of monocytes and neutrophilia with nuclear shift to the left become the more perceptible, consequently the total leucocyte counts rise above normal, exacerbation is induced, the progress of disease turns unfavorable and warning of bad prognosis is given. On the other hand, the nearer the main reaction arena is shifted to the IIIrd region, the more neutrophils and monocytes are decreased, lymphocytes are obviously increased, eosinophils and basophils are also increased and favorable course and good prognosis are to be expected.

The Basal Forms of Leucocyte Reaction in Infectious Diseases as Viewed from the Fields of Blood Defense Reaction

The defense mechanism against pathogenic microbes in an animal body consists of cell-bacterium reaction and antibody-antigen reaction, which have their respective and essentially different fields of action.

The selection of the field of crucial battle in the host-parasite struggle depends on the specificity of the infecting microbes. When the main battle-field is laid in the Ist region of Fig. 1, leucocytosis is the main event, when the decisive battle is fought in the IInd region, normo-leucocytosis is maintained and when it occurs in the IIIrd region, leucopenia is induced, as reflected in the picture of peripheral blood.

Here we must emphasize the importance of the basal forms of leucocyte reaction and inflammatory diseases. The cornerstones determining the structure of these basic forms are the respective characteristics of cell-bacterium reaction and antibody-antigen reaction — non-specificity in the former and specificity in the latter, against the infecting germs.

In infectious diseases, whether of leucocytosis, normo-leucocytosis or leuco-

penia case, first 1) neutrophils, 2) monocytes and such cells active in cell-bacterium reaction and then 3) lymphocytes, 4) eosinophils and 5) basophils that carry on the antibody-antigen reaction come in to take parts in the defense reaction.

The features of leucocyte reaction are more or less modified by the combination of the different times (reaction stages) and places (fields of reaction) of the main battle in the host-parasite struggle, but after all the main feature of reaction, whether of leucocytosis or leucopenia type, always shows a regular basal form, as shown in Fig. 6.

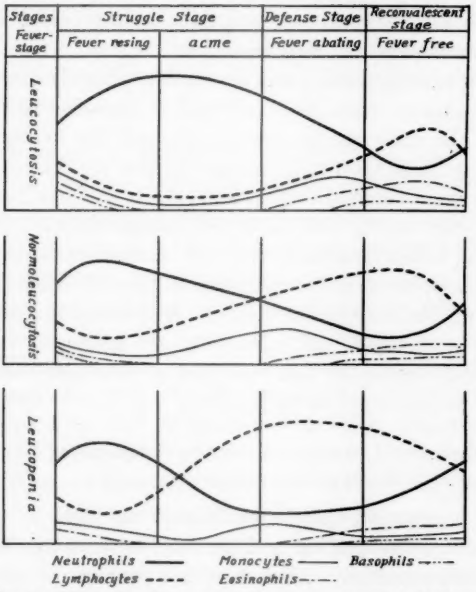

Fig. 6. Three basal forms of leucocyte reaction (schema).

1. The Basal Form of Leucocyte Reaction in Leucocytosis

The main defense reaction is enacted in the Ist region where the cell-bacterium reaction is in predominance; the percentage of neutrophils in the total leucocyte count markedly rises in the struggle phase but comes down gradually in the defense phase, lymphocytes are frankly decreased in acme but begin to increase in the fever-abating stage; in the convalescent stage, neutrophils keep on decreasing and the lymphocytes increasing, so that the two curves cross over; later on the former begin to increase and the latter to decrease again, so that the two curves recross and the normal constitution is reinstated, this double crossing is only narrow-spaced (Report 5, Figs. 2 and 4), and is often absent in

moderate and milder cases and cases with only short disease course (Report **5**, Figs. 1 and 3).

2. Ditto in Normo-leucocytosis

The main defense reaction for the host is fought out in the IInd region, where the cell-bacterium reaction and the antibody-antigen reaction are on equal footing; in the first half of acme the cell-bacterium reaction takes the lead, with increased neutrophils, but in the latter half of acme, lymphocytes make a marked spurt, so that the curves of these two types of cells cross each other in the defense phase; in the convalescent stage the lymphocytes increase further but after that begin to decrease, while neutrophils decrease and increase vice versa, so that the curves cross over again, and normalcy is restored. In milder cases, this double crossing is sometimes absent, but it tends to be less narrow-spaced than in leucocytosis.

3. Ditto in leucopenia

The field of main defense reaction is laid in the IIIrd region where the antibody-antigen reaction is in predominance, with a relative and later an absolute increase of lymphocytes; neutrophils increasing only transiently in the beginning of struggle phase (fever-rising stage); lymphocytes decrease temporarily at the out-set of struggle phase, but keep on increasing and increasing thereafter, so that neutrophil curve and lymphocyte curve come to crossing in acme (the latter half of struggle phase), as shown in Fig. 6; still, the former curve continues downwards and the latter upwards, the disparity becoming most marked in the fever-abating stage; in the convalescent stage, the neutrophil curve begins to rise and the lymphocyte curve to drop, to cross over again and normalcy is restored.

In leucopenic type diseases, the double cross is widest-spaced, and being always present excepting very mild cases, is known as the so-called Naegeli's typhoid cross ("Typhuskreuzung") (Report **5**, Fig. 5) and is accepted as a cross specific to typhoid fever, but this cross is not at all specific to leucopenic type diseases; only, it is most narrow-spaced in leucocytotic diseases, somewhat wider-spaced in normo-leucocytotic diseases and widest-spaced in leucopenic diseases. In leucopenic cases, the severer the disease, the earlier occurs the first crossing and the later the second, so that the ansal space between the two crosses becomes so much the larger.

Such reaction behaviors of monocytes, eosinophils and basophils remains essentially invariable in any type of leucocyte reaction; monocytes increase in the fever-abating stage (defense phase), eosinophils, besides increasing prominently in the convalescent stage, increase also in company of allergic phenomenon in acute exanthematic diseases, to detoxicate the allergic antigen,

and basophils begin to appear somewhat later than eosinophils and increase in the peripheral blood in convalescent stage and later.

CONCLUSION

Upon observing the leucocyte reaction from the angle of the fields of blood (vital) defense reaction in the adaptation process for internal environment, it is found that leucocyte reaction goes on in one of the three definite basal forms of leucocytotic, normo-leucocytotic and leucopenic types.

These 3 basal forms of leucocyte reaction are respectively characterized by the specificities of the first, the second and the third regions in which the main battle in the host-parasite struggle is fought out. The intensity of the leucocyte reaction depends on one hand on the specificity of the infecting microbes and their quantitative and qualitative differences, and on the other hand, on the individual difference of the host.

Consequently, it is recommended that the hitherto well-known Schilling's biological leucocyte curve ("Schilling's Biologische Leukocytenkurve") in infectious diseases should be radically revised, from the standpoint of the fields of blood defense reaction and the hematopoietic phases of the bone marrow, and the importance of the above 3 basal forms in leucocyte reaction should be stressed instead of the curve.

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New Methods of Laboratory Testing for the Sensitivity of the Uterus to Ovarian Hormones

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INTRODUCTION

A few investigators[1)-7)] have reported that abnormal sensitivity of the uterus to ovarian hormones exists among the endocrine disorders which are regarded as due to endocrinological disturbances. However, generally test measuring of hormones has been undertaken and the dosages of hormone preparations have been applied in diagnosing and treating endocrine disorders, and the problem on the sensitivity of the uterus has been nearly neglected. Abnormal sensitivity of the uterus is assumed to occur, either dependently or independently of endocrine disturbances of the sex hormones.

In the present studies, the author attempted to elucidate the problem of the sensitivity of the human uterus to ovarian hormones by the author's new idea and succeeded in finding new methods of laboratory testing for a clinical diagnosis of the sensitivity of the uterus to ovarian hormones (uterus-sensitivity hereafter).

Clinical cases of uterine amenorrhea caused by hyposensitivity of the uterus

The author will report three cases of uterine amenorrhea which is caused by hyposensitivity of the uterus to sex hormones to prove the existence of a hyposensitive uterus.

The first case: A twenty-six-year-old, unmarried, Japanese, non-gravida, complaining of primary amenorrhea.

On pelvic examination, external and internal genitalia were normal. Tentative curretage of endometrium was undertaken, which was unsuccessful to gain. A culture of tuberculous bacilli of a vaginal smear was negative. The basal body temperature (B.B.T. hereafter) revealed a typical biphasic curve (Fig. 1,a). The measured value of various kinds of steroid in the urine proved that the ovarian cycle was normal (Fig. 2,a). The thyroid and liver function

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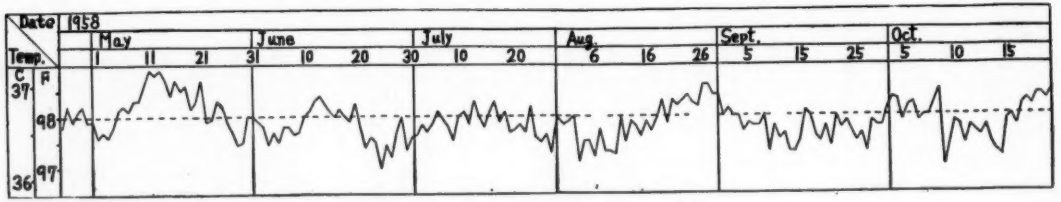

Fig. 1, a. B.B.T. of the first case.

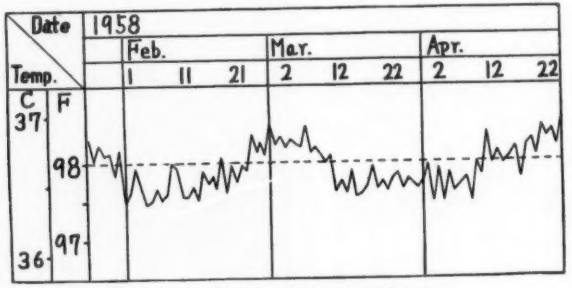

Fig. 1, b. B.B.T. of the second case.

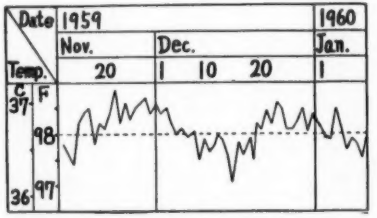

Fig. 1, c. B.B.T. of the third case.

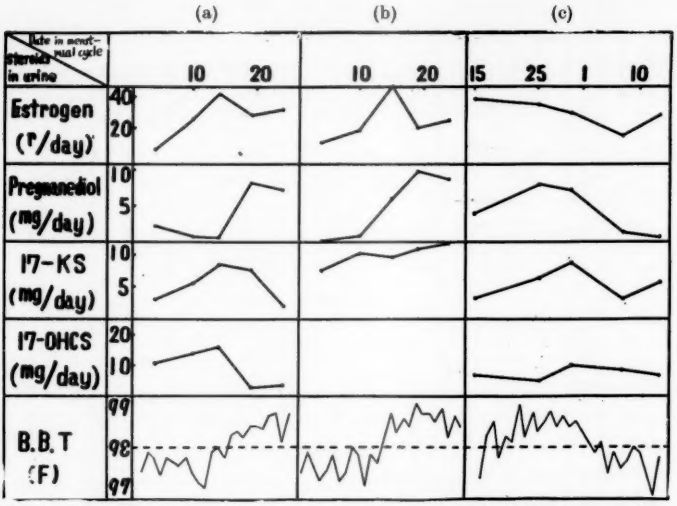

Fig. 2. B.B.T. and steroids in urine in three cases of uterine amenorrhea.
(a) the first case (b) the second case (c) the third case

were normal. In order to confirm the normal ovarian cycle, a tentative laparotomy was done on the high-temperature phase of her B.B.T. curve. Corpus luteum was found in the right ovary and endometrium was also found in the small rudimentary uterine cavity which showed no sign of menstrual bleeding. A histological picture of the endometrium showed no progestational one, but atrophic, in spite of the B.B.T. demonstrating the luteal phase (Fig. 3).

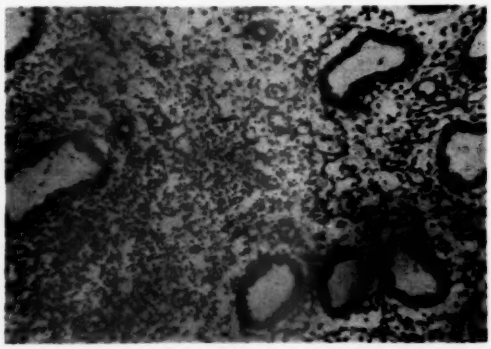

Fig. 3. Histological picture of the endometrium of the first case.

The second case: A twenty-four-year-old Japanese, para 1, gravida 1, complaining of secondary amenorrhea occurring after her delivery. On pelvic examination, the external genitalia and uterus were normal. The B.B.T. showed a typical biphasic curve (Fig. 1,b).

The measured values of various kinds of steroid in the urine proved a normal functioning of the ovary (Fig. 2, b). Endometrium was quite difficult to gain by curretage.

The third case: A twenty-three-year-old, Japanese, non-gravida, complaining of primary amenorrhea. On pelvic examination, the external genitalia and uterus were normal. The histological picture of endometrium demonstrated a proliferative phase, in spite of a high-temperature phase on the B.B.T. curve which was typically biphasic (Fig. 1, c), and the measured value of steroid in the urine proved that the ovarian cycle was normal (Fig. 2,c). ^{32}P-uptake test through endometrium (mentioned below) demonstrated the hyposensitivity of this uterus.

In all these three cases of uterine amenorrhea, the ovarian function was normal, but the uteri showed no reaction to ovarian hormone, that is, these uteri are supposed to be hyposensitive to ovarian hormone and deserve being referred to as hyposensitive uteri. There may be other hyposensitive uteri than these three cases of marked hyposensitivity, and new methods of laboratory testing are needed for the anomalous sensitivity of the uterus.

New methods of laboratory test for the uterus-sensitivity

The author devised new methods for laboratory testing for the uterus-sensitivity as follows:

I) Uterus-sensitivity test by ^{32}P–uptake through endometrium

A) Fundamental experiment (uterus function test)

1) Method

0.1 ml of sodium phosphate solution (pH 7.4) labelled with ^{32}P which was contained in the ratio of 2 μc per kg of body weight was infused into the uterine cavities of the subjects through the vagina with a syringe, then, 1 ml of blood was sampled from their cubital vein, 15, 30 and 60 minutes thereafter to measure the radio-activity with a syncillation counter (with head for β-ray) for 15 minutes. Their radio-activity was represented by counts per minutes (CPM hereafter) of arithmetric mean value.

2) Results (Fig. 4)

The subjects were divided into six groups. In normal subjects, the maximum value was attained in fifteen minutes ("rapid type"), the peak being higher in the cases in progestational phase (B) than those in the proliferative phase (A). The maximal values of senile uteri after menopause (C) and primary amenorrheic

uteri (D) were obviously lower than the normal, besides, retrogression of the peak was observed in the former ("slow type"). In secondary amenorrheic uteri (E), both types of the curve of ^{32}P-uptake were observed. Hypoplastic uteri (F) demonstrated the slow type of curve of ^{32}P-uptake, but their values were generally higher than in the cases of the senile uteri (C) and the primary amenorrheic uteri (D).

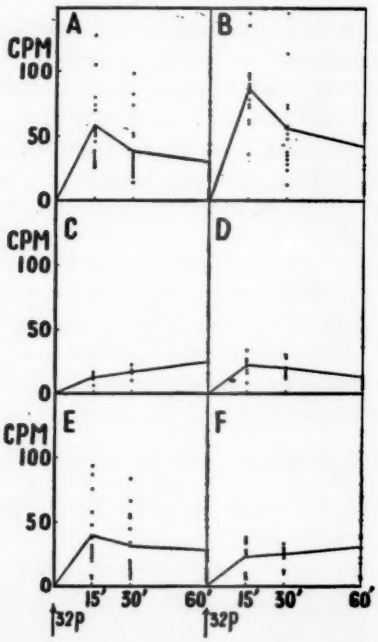

Fig. 4. Uterus function test by ^{32}P-uptake through endometrium.
• CPM per 1 ml of blood of the cases with various kinds of uterus, 15, 30 and 60 minutes after the infusion of ^{32}P into their uterine cavity.
—— Mean value of the above mentioned
A: Normal uterus (proliferative phase) B: Normal uterus (progestational phase) C: Senile uterus D: Primary amenorrheic uterus E: Secondary amenorrheic uterus F: Hypoplastic uterus

Evaluation of the results:

From the results of ^{32}P-uptake curve, a normal uterus was defined as follows: It demonstrates the rapid type of the curve, the maximal value of the curve being more than 20 CPM, and others were diagnosed as impaired uteri in their

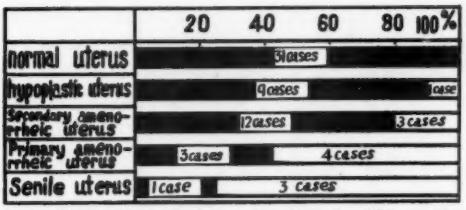

Fig. 5. Evaluation of uterus function by ^{32}P-uptake test in various kinds of uterus.
■ normal
□ impaired
} in uterus function.

function. Illustrative data evaluated from this criterion are presented in Fig. 5.

B) Uterus-sensitivity test

1) Principle: The value of the ^{32}P-uptake test may be affected by the change of hormonal circumstances. Therefore, the impaired function of the uterus, being detected by the ^{32}P-uptake test, can be produced either by an impaired uterus itself or insufficient secretion of sex hormones. In order to find an impaired uterus in its sensitivity, the following method was undertaken.

2) Method: Uterus function test by the ^{32}P–uptake curve was performed twice, directly before and twenty four hours after an intramuscular injection of 10,000 units of estradiol benzoate once a day for three successive days, and both values of the curve were compared.

3) Results (Fig. 6)

The subjects were divided into four groups. Most of normal uteri (A) represented marked elevation of the maximal values of the ^{32}P-uptake curves after the administration of estrogen comparing with before the administration. In the cases of secondary amenorrheic uteri (C) and hypoplastic uteri (D), some of them represented the same changes of value of the ^{32}P-uptake curve, but most of primary amenorrheic uteri showed no change in their ^{32}P-uptake curve (B).

Evaluation of the results:

The uterus which showed the CPM value of more than 20 and an increase of more than 20 % of CPM value at 15 minutes after infusion of ^{32}P, after the administration of estrogen, in comparison to that before the administration of estrogen was diagnosed as to be normally sensitive, and others were diagnosed as a hyposensitive uterus. Illustrative data in this connection are shown in Fig. 12.

II) Uterus-sensitivity test by measurement of oxygen consumption of uterine tissue

A) Method

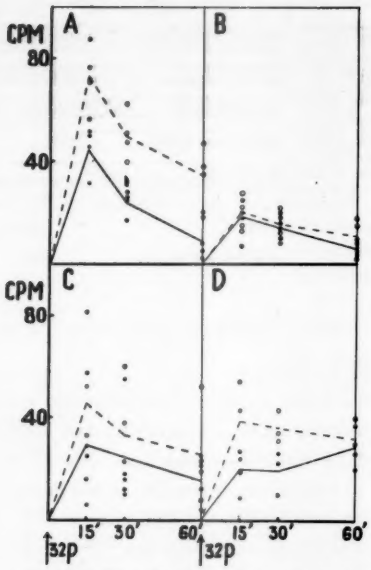

Fig. 6. Uterus-sensitivity test by ^{32}P-uptake through endometrium.
• CPM per 1 ml of blood after infusion of ^{32}P into the uterine cavity, before the administration of estradiol benzoate 1 mg daily for three days.
—— Mean value of the above mentioned.
o CPM per 1 ml of blood after infusion of ^{32}P into the uterine cavity, after the treatment above mentioned.
- - - Mean value of the above mentioned.
A: Normal uterus (proliferative phase) B: Primary amenorrheic uterus C: Secondary amenorrheic uterus D: Hypoplastic uterus

This test was begun from the third day after the completion of menstruation in the case of menstruating subjects, and from the arbitrary period in the case of amenorrheic subjects.

An endometrial specimen was sampled for the measurement of its oxygen consumption by curretage three times, i.e., the first, before the injection and the second, two days after the injection of 100,000 units of estradiol benzoate, which was followed by an injection of a mixed preparation of 250 mg of 17α-hydroxyprogesterone capronate and 10 mg of estradiol valerianate, the third, seven days after the second injection of the mixed preparation.

A myometrial specimen was taken at the same time of the second sampling of endometrium, by puncture-needle of Tsurumaru's type, modified by the author, and its oxygen consumption was measured.

The procedure of measurement of the oxygen consumption : A Cartesian diver manometer was used in the present study as a chief apparatus for measurement of oxygen consumption. The operation of this manometer was carried out essentially by Holter's method modified by S. Sugawara *et al.*[8], which represented good agreement with the result obtained by Warburg's method.

B) Results

Qo_2-value of endometrium (Fig. 7) : The Qo_2-values of normal uteri were found to be higher than those of amenorrheic uteri, especially, primary amenorrheic uteri, and increased by the administration of estrogen and decreased by progesterone, while the Qo_2-value of amenorrheic uteri was found to be low and showed little change by the administration of sex hormones.

Qo_2-value of myometrium (Fig. 8) : The Qo_2-value of uterine muscle was perceptibly low in the senile uterus after menopause and hypoplastic uterus, and notably high in a fibroid uterus in comparison to a normal uterus.

Evaluation of the results : The uterus in which the Qo_2-value of endometrium after the injection of estrogen was more than 2.6×10^{-2} μl/mg, was evaluated to be normally sensitive, and the others were evaluated to be hyposensitive.

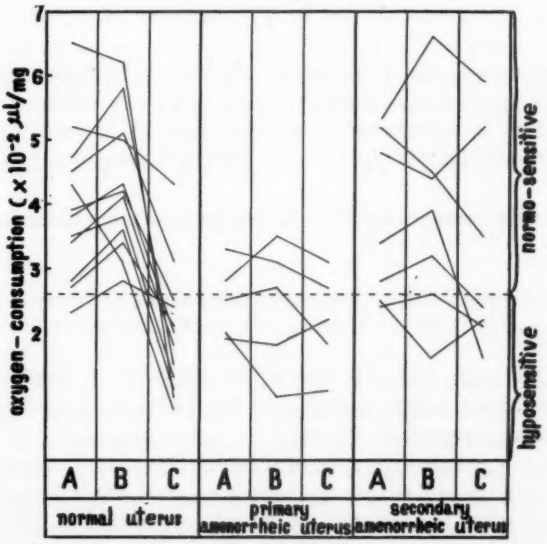

Fig. 7. Uterus-sensitivity test by Qo_2-measurement of endometrium.
A : before treatment.
B : two days after administration of estradiol benzoate 10 mg.
C : seven days after administration of estradiol valerianate 10 mg and 17α-hydroxyprogesterone capronate 250 mg.

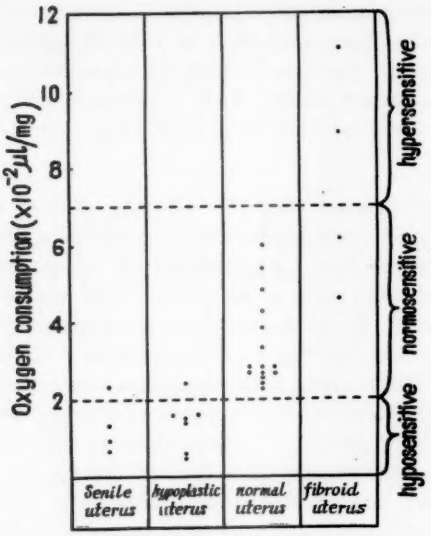

Fig. 8. Uterus-sensitivity test by Qo_2-measurement of myometrium.

The uterus showing Qo_2-value more than 7.0×10^{-2} μl/mg of uterine muscle tissue after the injection of estrogen was evaluated to be hypersensitive, and it was evaluated to be normosensitive and hyposensitive respectively, when the value was between 7.0×10^{-2} μl/mg and 2.0×10^{-2} μl/mg and below 2.0×10^{-2} μl/mg.

The illustrative data evaluated by these criteria mentioned above were shown in Fig. 12.

III) Uterus-sensitivity test by histological examination of the endometrium

A) Method

This test was begun immediately after the completion of menstruation in the subject with a normal menstrual cycle, and was begun in an arbitrary period in the subject of an amenorrheic uterus.

Endometrial specimens were sampled from subjects with normal menstrual cycles and of primary and secondary amenorrheic uteri, twice from each subject, i.e., before and after the injection of mixed preparation of 250 mg of 17α-hydroxyprogesterone capronate and 10 mg of estradiol valerianate.

These endometrial specimens were sampled by curretage from the medial portion of the uterine fundus of each subject.

B) Results (Fig. 9)

Most of the uteri with normal menstrual cycles were changed into the progestational phase from the proliferative phase in their histological picture after the injection of the mixed preparation of estrogen and progesterone, while most of the amenorrheic uteri showed to be remaining in the proliferative phase even after the injection of estrogen and progesterone.

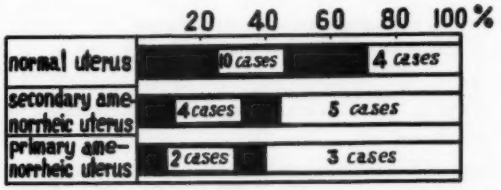

Fig. 9. Evaluation of uterus-sensitivity by histological examination for endometrium in various kinds of uterus.

■ cases changed into progestational phase seven days administration of estradiol valerianate 10 mg and 17α-hydroxyprogesterone capronate 250 mg (normosensitive uterus)

□ cases not changed into progestational phase seven days after administration of the above mentioned hormones (hyposensitive uterus)

Evaluation of the results: The endometrium of the uterus, in which any progestational finding in its histological picture was found after the injection of the mixed preparation of estrogen and progesterone, was evaluated to be normosensitive, otherwise to be hyposensitive.

The evaluated data from this criterion were shown in Fig. 12.

IV) Uterus-sensitivity test by crystallization of cervical mucus

A) Method

This test was begun from the third day after the completion of menstruation in subjects with a normal menstrual cycle, and from the arbitrary period in amenorrheic subjects.

Cervical mucus was taken with forceps, being careful not to stain with blood, and spread upon a slide and dried for microscopic examination.

This test was performed twice, i.e., directly before and 48 hrs. after intramuscular injection of 100,000 units of estradiol benzoate and their crystallizations were examined comparing both specimens. Their crystallizations were represented by an estimate of extent of the crystals varying from 0 to 5 in number, as shown in Table I.

Evaluation of the results: The uterus, of which cervical crystals showed difference of more than 2 in number of evaluation between two specimens was diagnosed as normosensitive and was diagnosed as hyposensitive, when the

Table I. Gradation of Crystallization of Cervical Mucus

Grade	Crystallization of cervical mucus	Indication
1	no crystal observed	0
2	non-typical crystal partly observed	1
3	non-typical crystal entirely observed	2
4	typical crystal partly observed	3
5	typical crystal entirely observed	4
6	nucleus of crystal appeared	5

difference was below 2. Illustrative data, classified in this connection are represented in Figs. 10 and 12.

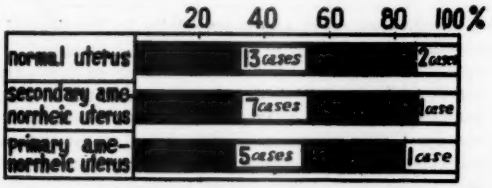

Fig. 10. Uterus-sensitivity test by crystallization of cervical mucus.
■ cases changed into high degree of crystallization of cervical mucus two days after administration of 100,000 units of estradiol benzoate (normosensitive)
□ cases not changed into high degree of crystallization of cervical mucus two days after administration of 100,000 units of estradiol benzoate (hyposensitive)

V) Uterus-sensitivity test by withdrawal-bleeding

The tests described above were originally devised by the author, while many reports concerning this withdrawal-bleeding test, have been published. The author modified this test to apply for the laboratory test of uterus-sensitivity as follows.

A) Method: The subjects were injected intramuscularly with the mixed preparation of 10 mg of estradiol valerianate and 250 mg of 17α-hydroxyprogesterone capronate, directly after the completion of menstruation of the subjects with a normal menstrual cycle or in the arbitrary period in amenorrheic subjects, and then, their withdrawal-bleeding was observed and was represented by the Kupperman's index modified by the author to apply for the test of uterus-sensitivity.

The withdrawal-bleeding index was obtained as follows: The time required for onset of bleeding in days, calculated from the day of injection of the mixed preparation of estrogen and progesterone to the first day of bleeding, was divided into the duration of withdrawal menstrual bleeding in days. The quotient ob-

tained was then multiplied by an estimate of the extent of bleeding, varying from 0 to 5, and 100. Thus, 1 bleeding referred to staining; 2 described a mild to moderate flow; 3 was considered as a diminished flow, but more than moderate; 4 designated a normal menstrual flow; and some cases were referred to withdrawal bleeding as being 5 (characterized by flooding or hypermenorrhea). The numerical figure of a withdrawal-bleeding index was determined by the following formula:

$$W.I. = \frac{D}{T} \times E \times 100$$

W.I.: Withdrawal-bleeding index
T : Time of onset
D : Duration
E : Extent
} of withdrawal-bleeding

B) Results

Evaluation of results: The uterus representing the withdrawal-bleeding more than 5 was diagnosed as normosensitive, and below 5 was diagnosed as hyposensitive. A summation of the result evaluated from this criterion was represented in Figs. 11 and 12.

All of the normal uteri were diagnosed as normosensitive from this test, and more than half of amenorrheic uteri were also shown to be normosensitive.

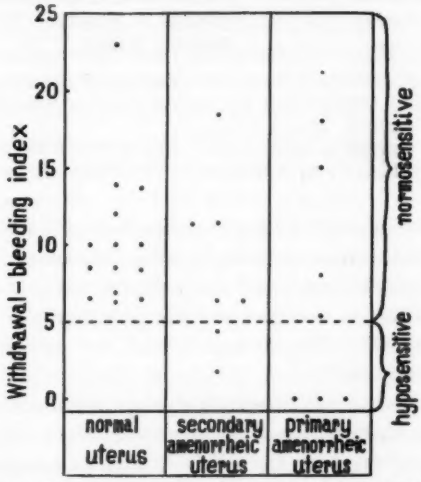

Fig. 11. Uterus-sensitivity test by withdrawal-bleeding after administration of estradiol valerianate 10 mg and 17α-hydroxy-progesterone capronate 250 mg.

DISCUSSION

Seeing the current study on the problem of the impaired function of the uterus, the uterus-sensitivity to sex hormone has been nearly neglected, and only

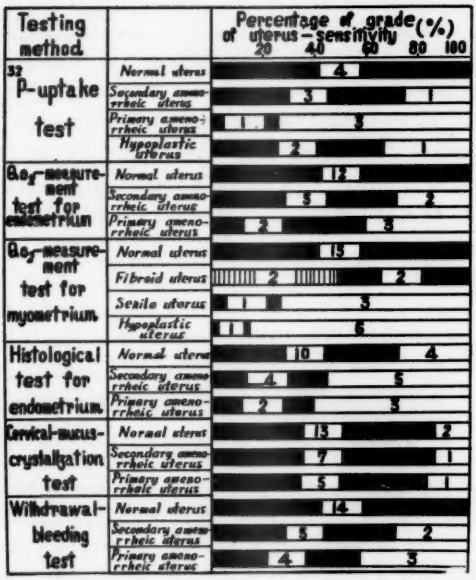

Fig. 12. Comparison of the evaluation of uterus-sensitivity obtained by various kinds of laboratory testing method.

hypersensitive
normosensitive
hyposensitive
(figures within the bars indicate the number of cases tested)

the withdrawal-bleeding test[9-12] has been performed for clinical diagnosis of amenorrhea of uterine origin. However, the numerical figure of the withdrawal-bleeding index has been based upon the extent of the bleeding depending on a subjective estimation and does not always reflect the uterine function as shown in Figs. 11 and 12. The author devised new methods of a laboratory test for the uterus-sensitivity.

1) Uterus-sensitivity test by ^{32}P-uptake through endometrium

Many investigators[13][14] have reported that the endometrial phosphate metabolism was accelerated by the administration of estrogen in experimental animals. In the present study, the value of ^{32}P-uptake curve can be considered to represent the level of phosphate metabolism of human endometrium. The measurement of the level of the phosphate metabolism using ^{32}P showed that the level was high in the normal uterus, but in the subjects of senile, primary amenorrheic and hypoplastic uteri and in some cases of secondary amenorrheic uteri, it was always lower than in normal uteri. This finding leads to the conclusion that the general metabolism is depressed in the endometrium of amenorrheic,

hypoplastic and senile uteri.

2) Uterus-sensitivity test by measurement of oxygen consumption

There are many reports,[15)16)] which showed the fluctuation of Qo_2-value with the menstrual cycle. In vitro, estrogen is considered to accelerate and progesterone to depress the Qo_2-value of human endometrium. In the present study, the change of Qo_2-value of endometrium and myometrium by the administration of estrogen and progesterone was expected to be an indicator of uterus-sensitivity to sex hormones.

It must be evidence for the normosensitivity to sex hormones of the uterus with a normal menstrual cycle that its Qo_2-value is high and elevated by the administration of sex hormones, and that the Qo_2-value in primary amenorrheic and some of secondary amenorrheic uterus is lower and not elevated must represent their hyposensitivity to the sex hormones.

In the case of uterine muscle tissue, the Qo_2-value after the administration of estrogen was notably high in the fibroid uterus which is assumed to be accelerated in its general metabolism, and lower in the senile and hypoplastic uterus which is supposed to be depressed in its general metabolism. These results prove this test to be reasonable as a laboratory test of the sensitivity of uterus muscle.

3) Uterus-sensitivity test by histological examination of endometrium

It is well known that endometrium changes its histological picture according to the cyclic changes of ovary, and conversely, endometrial dating from its histological picture is fairly accurate comparing with B.B.T. and menstruation.[7)]

In the present study, the presence of histological changes showing progestational picture of endometrium, as examined seven days after administration of estrogen and progesterone, was used as the indicator of the uterus-sensitivity.

The percentage of the uteri which showed the progestational picture was a little higher in the normal uterus than in the amenorrheic uterus, but all of the normal uteri did not always represent the histological change. This test needs more improvement in the dosage of sex hormones.

4) Uterus-sensitivity test by the crystallization of cervical mucus

Crystallization phenomenon of cervical mucus was first demonstrated by Papanicolau[18)19)] in the dried smear of cervical mucus, which was confirmed to be strengthened by the administration of estrogen and weakened by progesterone by Bergman,[20)] Campos da Paz,[21)22)] and others.

The author attempted to employ the crystallization phenomenon of cervical mucus as an indicator of uterus-sensitivity.

In this test, no obvious difference was observed among normal, secondary and primary amenorrheic uteri in the ratio of normosensitive and hyposensitive uterus. The result suggests that anomalous sensitivity of cervical endometrium has no correlation with the length of the duration of amenorrhea.

SUMMARY

In order to elucidate the problem of uterus-sensitivity to ovarian hormones, the following experiments were carried out.

1) The author proposed a new desease "Hyposensitive uterus", provisionally denominated, and three cases of it were reported. This desease is characterized by irresponsiveness of the uterus to ovarian hormones, regardless of the normal function of ovary, confirmed by typical biphasic B.B.T. curve, normal value of estrogen and pregnanediol excretion in the urine and confirmation of corpus luteum by laparotomy.

2) Various kinds of methods of laboratory testing of uterus-sensitivity were devised to make diagnosis of anomalous sensitivity of uterus. A summation of data examined by these tests leads to the conclusion that uterus-sensitivity test by the measurement of oxygen consumption of endometrium and myometrium was most convenient and valuable in clinical use.

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